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Supplementary Materials for

A human gut *Faecalibacterium prausnitzii* fatty acid amide hydrolase

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Other Supplementary Materials for this manuscript include the following:

Tables S1 to S11 (.xlsx)

Materials and Methods

Gnotobiotic mouse husbandry

All mouse experiments were performed using protocols approved by the Washington University Animal Studies Committee. All experimental animals were included in our analyses; no inclusion or exclusion criteria were applied). Germ-free male C57BL/6J mice were maintained in plastic flexible film gnotobiotic isolators (Class Biologically Clean Ltd., Madison, WI) at 23 °C under a strict 12-hour light/dark cycle (lights on at 0600 h). Animals were co-housed in cages (n = 4-6 animals/experimental group; maximum of 5 animals/cage) that contained autoclaved bedding (Aspen Woodchips; Northeastern Products) and 'shepherd shacks' to facilitate their natural nesting behaviors and to provide an activity center for environmental enrichment. At three weeks of age, mice were weaned onto an autoclaved, low-fat, plant polysaccharide-rich mouse chow (Envigo, Catalog No. 2018S) that was administered *ad libitum*. They were maintained on this diet until two days prior to initiation of the experiment, when they were switched to a MDCF diet. To verify the germ-free status of the animals, fecal samples were collected prior to colonization and analyzed using culture and culture-independent assays (*11*).

Animals were colonized at 4-5 weeks of age with defined consortia of bacterial strains using a flexible plastic-tipped oral gavage needle (Fisher) and euthanized by cervical dislocation on experimental day 28 without prior fasting. The consortium consisted of cultured, sequenced human gut bacterial strains isolated from healthy 6-to-24-month-old Bangladeshi children (*11*) and maintained as frozen glycerol stocks at -80 °C. A frozen glycerol stock of each bacterial strain was thawed in an anaerobic chamber (Coy Laboratory Products) and inoculated into LYBHI medium. Monocultures were incubated under anaerobic conditions at 37 °C for 2 days. On the morning of gavage, the cultures were pooled at an equivalent optical density at 600 nm and sealed in Crimp-Top EZ Vials (Wheaton). The vials were sterilized with Clidox (Pharmacal) for 30 minutes, introduced into the gnotobiotic isolators, and a 200 µL aliquot administered to the animals using a plastic-tipped oral gavage needle.

Cryopreserved stocks of *F. prausnitzii* Bg7063 (*11*) and *F. prausnitzii* TS M3092 (*10*) (described in **Fig. 1**) were struck onto LYBHI-agar plates and the plates were incubated overnight at 37 ºC in an anaerobic chamber (Coy Laboratory Products; atmosphere 75% N_2, 20% CO_2, 5% H_2). Single colonies were picked, inoculated into 6 mL of liquid LYBHI medium, and incubated under anaerobic conditions for 1 day. Turbid cultures were transferred to 15 mL conical tubes, the tubes were tightly sealed, and cells were pelleted by centrifugation. DNA was recovered from the resulting cell pellet using a high molecular weight genomic DNA extraction kit (MagAttract HMW, Qiagen). Purified DNA was prepared for long-read sequencing using the SMRTbell Template Prep Kit (v2.0, PacBio) and Barcoded Adapter Kit (PacBio) and whole genome sequencing was performed [PacBio Sequel System]. Sequencing reads were demultiplexed and converted from binary alignment map (BAM) to fastq format (SMRT Tools software, v5.1.0 or 6.0.0). Long reads were assembled using Flye (v2.8.1) (*75*). Assembly quality statistics were generated using Quast (v4.5) (*76*). Open reading frames were identified and annotated using Prokka (v1.14) (*77*). Annotated *F. prausnitzii* Bg7063 genome files are available at https://zenodo.org/records/14013545).

For experiments involving treatment with the NAPE-PLD inhibitor LEI-401, a 3 mg/mL stock solution was prepared in DMSO. Aliquots were mixed with Tween-80 and deionized water (1:1:3) by pipetting. The resulting suspension was filter sterilized by passing through a 0.22 µm filter (Millipore Sigma) in a sterile hood, placed in Crimp-Top EZ Vials (Wheaton) and the vials were sealed by crimping. The vials were subsequently sterilized with Clidox (Pharmacal) for 1

hour, introduced into the gnotobiotic isolators, and administered to mice using a plastic-tipped oral gavage needle.

***F. prausnitzii* Bg7063 monoculture experiment described in Fig. 1C**

For the *in vitro* assays performed with *F. prausnitzii* Bg7063, a frozen glycerol stock was thawed in an anaerobic chamber and inoculated into pre-reduced LYBHI liquid medium at 37 °C for 48 h. On the evening prior to the experiment, a 500 µL aliquot was used to inoculate 100 mL of LYBHI broth. After a 16 h incubation at 37 ºC, a 3.3 mM stock solution of deuterated PEA (PEA-d_4; Catalog number 10007824; Cayman Chemical, MI) in ethanol was diluted with ethanol to create a 500 µM working stock which was then introduced into the anaerobic chamber. Triplicate 5 mL exponentially growing cultures received 100 µL of the PEA-d_4 working stock. Ethanol-treated LYBHI broth alone and ethanol-treated cultures served as reference controls. Samples were incubated for 1 h at 37 °C without agitation; 100 µL aliquots of the culture ($1.2 \times 10^9 \pm 1 \times 10^8$ CFU/mL) were then withdrawn and the reaction was quenched with 500 µL methanol. The quenched samples were removed from the anaerobic chamber and cellular homogenates were prepared as described in '*Sample extraction for mass spectrometric analyses*' below. A 150 µL aliquot of the homogenate was used to measure protein concentration [Pierce Micro BCA (bicinchoninic acid) Protein Assay Kit (Thermo Scientific)].

Comparison of NAE hydrolytic activity among *Faecalibacterium* strains (Fig. 1B)

Frozen stocks of *F. prausnitzii* Bg7063 and two other *Faecalibacterium* strains (*F. prausnitzii* TS M3092 and *F. duncaniae* DSM 17677) were thawed and cultured under anaerobic conditions. Their identities were verified by sequencing amplicons generated by PCR from the full-length 16S rDNA gene. On the evening prior to the experiment, OD_{600} measurements were used to normalize cell densities across the three monocultures. After a subsequent 16 h incubation at 37 ºC, monocultures were confirmed to have achieved an equivalent cell density (OD600) and the cultures were treated with PEA-d4. Samples were processed as described in the preceding paragraph. Additionally, equal numbers of *F. prausnitzii* Bg7063 cells and either *F. prausnitzii* TS M3092 or *F. duncaniae* DSM 17677 cells were pooled together such that the total number of cells was equivalent across samples. These pooled samples, as well as monoculture controls were treated with PEA-d4, incubated at 37 ºC for 1h in the anaerobic chamber, and the NAE hydrolytic activities of the *Faecalibacterium* species alone, or the pooled samples were determined.

Purification of palmitoylarginine (Fig. 2)

Six liters of a monoculture of *F. prausnitzii* Bg7063 were incubated for 1h in LYBHI medium containing 500 µM PEA at 37 ºC under anaerobic conditions for 1h. The entire culture was lyophilized (Triad system; Labconco) at -20 °C for 4 days. One liter of methanol was added to the lyophilized culture and the mixture was vortexed vigorously. The resulting mixture was aliquoted into twenty 50 mL glass tubes which were centrifuged at 3,220 x g to precipitate protein and cellular debris. The resulting supernatants was subjected to preparative HPLC using an Agilent 1260 HPLC system coupled to a fraction collector. Two rounds of protein purification were performed. The first round used a XBridge Prep C18 column [19 mm (internal diameter) x 50 mm (length), 5 µm (particle size), Waters Corp., Milford, MA] and a gradient program consisting of 70% to 100% mobile phase B from 0 to 10 minutes (mobile phase A, 0.1% formic acid in water; mobile phase B, acetonitrile/0.1% formic acid). Fractions collected from 7.5 to 8.5

minutes were combined, dried and subjected to a second round of purification employing a longer XBridge Prep C18 column (19 x 150 mm, 5 μm) and the following gradient program: 58% to 70% mobile phase B from 0 to 20 minutes; 70% to 100% mobile phase B from 20 to 30 minutes. The fraction collected at 26 minutes contained palmitoylarginine at a purity of 85% by LC-QqQ-MS.

HPLC-purified palmitoylarginine was reconstituted in 500 μL of 99.8% deuterated methanol (MeOD). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III 800 MHz spectrometer equipped with a 5 mm QCI (HC/N-P) cryoprobe and Z-axis gradient (Billerica, MA). Chemical shifts are reported in ppm relative to the reference signal and coupling constant (*J*) values are reported in hertz (Hz). **^{1}H NMR:** (800.1 MHz, MeOD) d 0.90 (t, *J*=7.2 Hz, 3H), 1.31-1.34 (brs, 24H), 1.62 (m, 2H), 1.71 (m, 2H), 1.78 (m, 1H), 1.96 (m, 1H), 2.28 (m, 2H), 3.25 (m, 2H), 4.40 (m, 1H), 7.50 (brs, 1H), 8.27 (d, *J*=7.5 Hz, 1H). **^{13}C NMR:** (201.2 MHz, MeOD) d 13.2 (1C), 22.5 (1C), 25.1(1C), 25.6 (1C), 28.3(1C), 29.0 (1C), 29.1 (2C), 29.4 (6C), 29.3 (1C), 31.7 (1C), 35.5 (1C), 40.6 (1C), 51.7 (1C), 157.2 (1C), 173.7 (1C), 175.2 (1C).

Sample extraction for mass spectrometric analyses

Cecal contents and feces collected from mice were immediately frozen in liquid nitrogen after collection, and maintained in -80 °C. Each sample was combined with a volume of ice-cold methanol equal to 19 times the weight of the sample as well as 1 volume by weight of PEA-d_4 (200 μg/mL). The mixture was subsequently homogenized by agitation (BioSpec Mini-Beadbeater, Atkinson, NH) for 4 minutes at room temperature. In the case of monocultures, 100 μL of a vortexed monoculture sample was mixed with 400 μL methanol. For all sample types, the mixtures were centrifuged (12,000 × g for 5 minutes at 4 °C). A 500 μL aliquot of each supernatant was transferred to a new tube and dried in a centrifugal evaporator (LabConco CentriVap). Dried samples were resuspended in 100 μL of 50% methanol and centrifuged at 12,000 × g for 1 minute at 4 °C to ensure that no particulate matter was carried forward to the next step. An 80 μL aliquot of each supernatant was then transferred into an Agilent sample vial (with fixed insert) and stored at 4 °C prior to injection into a mass spectrometer.

Untargeted LC-Qtof-MS

Untargeted metabolomic analyses were performed using an Agilent 1290 LC system coupled to an Agilent Model 6545 Qtof mass spectrometer (Santa Clara, CA). Reversed phase separation was achieved on a BEH C18 column (2.1 x 150 mm, 1.7 μm, Waters Corp., Milford, MA) that was heated to 35 °C. For analyses carried out in the positive ESI mode, the mobile phase consisted of 0.1% formic in water (A) and 0.1% formic acid in acetonitrile (B). For analyses in the negative mode, the mobile phase consisted of 5 mM ammonium bicarbonate in water (mobile phase A) and 5 mM ammonium bicarbonate in acetonitrile/water (95:5 v/v) (mobile phase B). The following gradient program was applied: 0 to 14 minutes, mobile phase B eluted from 5% to 100%, followed by 3 minutes at 100% of mobile phase B (flow rate of 0.3 mL/minute). Normal phase separation was conducted on a Cortex HILIC column (2.1 x 150 mm, 1.6 μm, Waters Corp., Milford, MA) (heated to 45 °C) and the following gradient program: 10 mM ammonium acetate in acetonitrile/water (5:95 v/v; mobile phase A); 10 mM ammonium acetate in acetonitrile/water (95:5 v/v; mobile phase B);1–60% phase A over 17 minutes; flow rate 0.4 mL/minute; followed by a 4 minute equilibration. Data were collected in the range from m/z 50 to 1000, and m/z 50 to 650 for MS full-scan analysis and MS/MS analysis, respectively.

The key parameters of Qtof were set as follows: nozzle voltage, 1000 V and 1500 V for positive and negative ESI modes, respectively; capillary voltage, 3000 V for positive mode and 3500 V for negative mode; drying gas, N_2; drying gas flow rate, 10 L/min; collision gas, high purity N2; drying gas (N_2) temperature, 325 °C; vaporizer/sheath gas temperature, 350 °C; sheath gas flow rate, 12 L/min. To ensure accurate mass measurements, reference masses m/z 121.0509 and 922.0098 for the positive ESI mode and 119.0362 and 980.01637 for the negative mode were automatically delivered using a dual ESI source during analyses. The mass accuracy of our LC-MS system was generally better than 4 ppm. Samples were randomly analyzed.

The resulting raw data sets were deconvoluted using MassHunter Profinder B.08.00 software (Agilent Technologies, Santa Clara, CA) which generated a list of molecular features. These features were subsequently filtered using in-house scripts to identify those that were significantly higher or lower in the experiment groups compared to the control group. These features were fragmented by targeted MS/MS with collision energy from 0 to 40 V. Final metabolite identification was performed by co-characterization with standards.

Targeted LC-QqQ-MS of fatty acids and *N*-acylamides

***Fatty acids* -** Five microliters of sample extract (see above) were injected into a 1290 Infinity II UHPLC system coupled to a Model 6470 Triple Quadrupole LC/MS system equipped with a Jet Stream electrospray ionization source (Agilent Technologies). Chromatographic separation was performed on a ZORBAX Extend-C18 column (2.1 x 50 mm, 1.8 μm; Agilent Technologies) using two buffers. Mobile phase A was 70% acetonitrile, 0.5 mM ammonium acetate. Mobile phase B was 99% acetonitrile, 1% 0.5 mM ammonium acetate. The flow rate was 0.4 ml/min, and the gradient conditions were as follows: 0-100% B from 0-10 minutes followed by a two-minute equilibration. Mass spectra were acquired in negative ESI mode and fatty acids were quantified by $[M-H]^-$.

***N-acylamide*s –** The analysis was carried out using the same equipment used for quantifying fatty acids with the following gradient conditions: mobile phase A (water + 0.1% formic acid); mobile phase B (acetonitrile + 0.1% formic acid); 20-100% mobile phase B (0-7 minutes); 100% mobile phase B (7-8 minutes); 100-20% mobile phase B (8-9 minutes); flow rate of 0.3 mL/minute). Mass spectra were acquired in positive multiple reaction monitoring (MRM) mode. Quantification transitions and mass spectrometric parameters were determined by using Agilent Optimizer.

Purification of FAAH activity from *F. prausnitzii* Bg7063

A 50 mL monoculture of exponentially growing *F. prausnitzii* Bg7063 was centrifuged (5,000 × g for 10 minutes). The resulting pellet was extracted by 2 mL of 1% NP-40 with a 10 second sonication (Sonifier 250; Branson) and then centrifuged (12,000 × g for 5 minutes at 4 °C). The extract was fractioned using an ÄKTA pure chromatography system (GE Healthcare) equipped with a HiTrap Q HP anion exchange chromatography column (5 mL column size, GE Healthcare). Binding buffer was prepared by dissolving Tris in deionized water to a final concentration of 20 mM, and the pH was adjusted to 8.0 with HCl or NaOH. The elution buffer was prepared similarly, with addition of NaCl to a final concentration of 1 M. Both buffers were filtered through a 0.22 μm membrane filter and degassed before use. The ÄKTA pure system was first equilibrated with the binding buffer at a flow rate of 1 mL/minute until the absorbance baseline was stable. The protein sample was then loaded onto the HiTrap Q HP column. The column was washed with the binding buffer until the absorbance at 280 nm returned to the

baseline level, thereby ensuring that all unbound proteins were washed off. The bound proteins were eluted using a linear gradient of the elution buffer; the gradient was started at 0% elution buffer and increased to 100% over 1 h at a flow rate of 1 mL/minute. Elution of proteins was monitored by measuring the absorbance at 280 nm. Fractions were collected during the elution step, and each fraction was tested for PEA degradation activity (see above for the LC-QqQ-MS method).

The fraction from anion exchange chromatography demonstrating the highest PEA degradation activity was subjected to proteomic analysis using a protocol described in ref. *78*. Briefly, the fraction was precipitated using acetone-TCA and the protein pellet was reconstituted in 8M urea. Samples underwent reduction with 20 mM dithiothreitol at 37 °C for 1 h, followed by cysteine alkylation (carried out in the dark by incubation with 80mM iodoacetamide for 45 minutes). Following an overnight incubation at 37 °C with 600 ng of trypsin, the resulting peptides were desalted through solid-phase extraction on a C18 Spin column and eluted with 0.1% formic acid in 80% acetonitrile. A nanoElute connected to a timsTOF Pro2 Mass Spectrometer (Bruker Daltonics) was used for LC-MS/MS analysis of the peptides. Samples were loaded onto a capillary C18 column (15 cm length, 75 μm inner diameter, 1.9 μm particle size, 120 Å pore size; Bruker Daltonics), with the flow rate maintained at 300 nL/minute. Solvent A (0.1% formic acid in water) and Solvent B (0.1% formic acid in acetonitrile) were used to separate peptides over a 100-minute-long analytical gradient, transitioning from 2% acetonitrile/0.1% formic acid to 35% acetonitrile/0.1% formic acid (total gradient period = 120 minutes). The timsTOF Pro2 was operated in PASEF mode, acquiring MS and MS/MS spectra ranging from 100 to 1700 m/z. The inverse reduced ion mobility 1/K0 was set to 0.60−1.60 V·s/cm2 over a ramp time of 100 ms. Data-dependent acquisition involved 10 PASEF MS/MS scans per cycle with almost a 100% duty cycle. Protein tandem MS data were compared against the *F. prausnitzii* proteome using MaxQuantv2.1.0.0.

Expression and purification of *F. prausnitzii* FAAH from *E. coli* (for experiments described in Figs. 2 and 3)

FAAH protein expression was performed by Genscript USA (Piscataway, NJ). Briefly, a signal sequence-deficient (residues 2-33) *F. prausnitzii* FAAH gene was synthesized (NdeI--ATG--His tag--protein --Stop codon--HindIII) and subcloned into pET-30a(+). BL21 Star™ (DE3) competent cells were mixed with plasmid DNA, incubated on ice, heat-shocked, and then incubated with LB medium. Expression of the FAAH was induced with 0.5 mM IPTG at 15°C for 16 hours. Cells were harvested by centrifugation. Cell pellets were resuspended with 1% NP-40 followed by sonication. His-tagged FAAH was purified using Ni affinity chromatography (Rio-Rad, Profinity™ IMAC Resin, Ni-charged).

Measurement of bifunctional *N*-acylamide hydrolase/synthase activity (Figs. 2 and 3)

All reactions took place in a 100 μL solution consisting of 50 mM Tris-HCl and 150 mM NaCl at pH 7. For assessing hydrolase activity, the test *N*-acylamide was added to the reaction mixture at concentrations ranging from 10 to 100 μM. To evaluate synthase activity, a fatty acid (100 μM), and an amine (100 mM) were added. Lysate or purified enzyme was quantified by BCA assay (Thermo Fisher Scientific) and then adjusted to 200 μg/mL and 10 μL was added to assays. Following a 60-minute incubation at 37 °C (for reaction rate measurements, incubation times were 2 minutes, 5 minutes, and 20 minutes), each reaction was halted by adding 1 mL of

methanol. The solution was then pelleted at 14,000 x g. The *N*-acylamide and its corresponding acyl chain were measured using the LC-QqQ-MS-based method described above.

FAAH inhibitor studies

Four human FAAH inhibitors tested (PF-750, PF-3845, URB597, CAY10435) and the cysteine peptidase inhibitor E-64 were each dissolved in DMSO at 1 mM and then diluted to 100 µM in ethanol. They were further diluted to 5 µM in reaction buffer (50 mM Tris-HCl and 150 mM NaCl with pH at 7) as working stock. The inhibitor assay has been described previously (*33*). Briefly, for each inhibitor, 10 µL of purified *F. prausnitzii* FAAH (100 µg/ml) and 10 µL of inhibitor (achieving 2.5 µM in the mixture; note that all of the four inhibitors have been reported to fully inhibit human FAAH at 1 µM) were preincubated for 1 h at 37°C. The PEA-d_4 degradation assay described above was performed. Each reaction was stopped after a 1 h incubation at37 °C by adding 1 mL of methanol. PEA-d_4 levels were then quantified by LC-QqQ-MS.

GPCR screen of NAAAs by DiscoverX assay

C18:1-Arg, C18:1-His, and C16:0-Arg were synthesized by Aldlab (Boston, USA) with 99% purity confirmed by NMR. The purified compounds were submitted to DiscoverX for testing in a cell-based assay described in ref. *49*. The assay encompassed a panel of 18 nuclear hormone receptors (nhrMAX), as well as 73 GPCRs (orphanMAX). The agonist activity of each compound tested (at a concentration of 10 µM) for each GPCR was defined using chemiluminescence as an output indicator of β-arrestin recruitment. 'Activity percentage' was calculated relative to the baseline value (0% activity) and the maximum value triggered by a known ligand (100% activation). Agonist as well as antagonist activities were defined for each of the NHRs tested. DiscoverX provides an empirical threshold value of 30% or 50% for NHR agonist/antagonist (and GPCR agonist), respectively; activity or inhibition exceeding the threshold is deemed "suggestive that the interaction between the compound and the receptor could be biologically significant".

NAAA dosing experiment in gnotobiotic mice

C18:1-Arg and C18:1-His were chemically synthesized by Aldlab (Boston, USA). The purity of the product was confirmed to be 99% by NMR. To determine the dose of NAAAs needed to achieve concentrations in the 100 ng/g range in the distal gut, we conducted a pilot experiment in which C18:1-Arg was administered by gavage to 4- to 5-week-old, male C57BL/6J mice (n=3). Two stock solutions were prepared; either 4 mg or 12.5 mg of C18:1-Arg was dissolved in 0.4 mL of 1:1 methanol-ethanol solvent and then mixed with 5.6 mL of vehicle [vehicle = 0.5% methylcellulose; catalog number M0262; Sigma) + 0.2% Tween-80]. The resulting suspension was filter sterilized by passing through a 0.22 µm filter (Millipore Sigma) in a sterile hood and sealed in Crimp-Top EZ Vials. The vials were sterilized with Clidox (Pharmacal) for 30 minutes, introduced into the gnotobiotic isolators, and a 0.3 mL aliquot was administered as a single oral gavage to each animal (dose = 8 mg/kg/d or 25 mg/kg/d; n=3 mice/dose). Fecal samples were collected at 2-, 4-, 8-, and 24-hours post-gavage and levels of C18:1-Arg were measured using the targeted LC-QqQ-MS method described above. Two weeks after the initial gavage treatment was repeated; this interval between dosing was chosen to ensure washout. Mass spectrometry data confirmed the absence of detectable fecal C18:1-Arg before administration of the first dose. C18:1-Arg was initially detected in feces 8 hours post-gavage and reached maximum levels of

97.4 ± 14.2 ng/g and 262 ± 19.3 ng/g and in samples collected 24 h post-administration of the low and high doses, respectively.

Assaying the effects of NAAAs in germ-free mice (Fig. 4)

Experimental design and dosing - Four experimental groups of 4- to 5-week-old, male C57BL/6J mice were maintained germ-free in a single gnotobiotic isolator (5 mice/cage/group). Two days prior to the experiment, all animals were switched from an autoclaved, low-fat, plant polysaccharide-rich mouse chow (Envigo, Catalog No. 2018S) to the MDCF-2 diet (*14, 15*) which had been sterilized by gamma irradiation (30-50 KGy). On the morning of experimental day 1, an 8 mg/kg/day dose of OEA, C18:1-Arg and C18:1-His was prepared and administered to each group of animals by oral gavage. One experimental group that received vehicle alone (i.e. the ethanol/methylcellulose/Tween-80 mixture) served as reference controls. Animals in each experimental group subsequently received an oral gavage of the NAAA daily at 10AM for 3 additional days. A final gavage was performed at 4PM on the day prior to euthanasia. On experimental day 5, mice were euthanized by cervical dislocation without prior fasting. Aliquots of portal blood, plus liver and multiple gut segments (jejunum, ileum, and colon) were rapidly recovered, flash frozen in liquid nitrogen and stored at -80 °C.

RNA-Seq analysis of gene expression in the intestine and liver - Total RNA was isolated using the 'RNeasy Plus Universal Mini' Kit (Qiagen, Hilden, Germany; Catalog No. 74104) from flash frozen intestinal segments (jejunum, ileum, colon) and liver collected at euthanasia from germ-free animals treated with NAAAs at frozen at -80 ºC. Frozen samples were transferred to 2 mL Lysing Matrix F tubes (MP Biochemicals, CA) that contained 1.6 mm Al_2O_3 particles and 1.6 mm SiC particles (MP Biochemicals) plus 600 µL of Buffer RLT (RNeasy kit) supplemented with 143 mM β-mercaptoethanol. Samples were then lysed by agitation (BioSpec Mini-Beadbeater, Atkinson, NH) for 4 minutes at room temperature. The resulting lysates were centrifuged (12,000 × g for 5 minutes at 23 °C) and a 400-µL aliquot of the supernatant was transferred to a new tube and mixed with 400 µL of 70% ethanol. A 700 µL aliquot of the resulting mixture was transferred to a RNeasy spin column and centrifuged (12,000 × g for 30 seconds at 23 °C). Residual genomic DNA was eliminated by performing an on-column DNase digestion (30 Kunitz Units DNase I/ RNeasy column; Catalog No. 79254; Qiagen) for 15 minutes at room temperature. Columns were washed with buffers RW1 and RPE in the RNeasy kit and purified total RNA was eluted in 100 µL nuclease-free water (Ambion). RNA integrity and fragment size distribution were determined using a BioAnalyzer RNA 6000 Pico kit (Agilent Genomics). A 200 ng aliquot of the purified RNA preparation was then depleted of ribosomal RNAs using biotinylated, target-specific oligos combined with Ribo-Zero rRNA removal beads (rRNA Removal Mix – Gold, Illumina).

The rRNA-depleted sample was then fragmented, and 10 ng aliquots and random hexamers were used for synthesis of cDNA (TruSeq Stranded Total RNA kit). Following adenylation of the 3' ends of the blunt double-stranded DNA fragments and ligation of indexing adapters, sequencing libraries were prepared using PCR primers directed to the ends of the adapters. Libraries pooled, normalized and sequenced (Illumina NextSeq 500 instrument; 75-nt-long unidirectional reads; $28.1 \times 10^6 \pm 3.3 \times 10^6$ reads/sample). Adapters were removed from the FASTQ reads and low-quality 3' ends were trimmed using Trim Galore (version 0.6.1) (*79*), with default parameters aside from a Phred score of 28. Release 104 of the *Mus musculus* primary assembly genome was downloaded from Ensembl (GRCm39.104) and an index file for the STAR aligner was generated (STAR version 2.7.2b) (*80*) with default parameters aside from

"--*sjdbOverhang* 74" for unidirectional reads. Quality-controlled reads were mapped to the mouse genome using STAR with default parameters aside from: "--*outFilterMatchNmin* 25", "--*sjdbOverhang* 74", "--*outSAMtype* BAM Unsorted SortedByCoordinate", "--*quantMode* TranscriptomeSAM GeneCounts", to generate binary BAM-formatted alignment files. Quantification of genomic features (the number of uniquely mapped reads per feature) was performed using the featureCounts algorithm (subread v2.0.0) (*81*) with the non-default parameter "-*s* 0" (i.e., reads with no strand information). Raw transcript count data were analyzed using DESeq2 (R version 3.5.1) (*82*) to identify differentially expressed genes. Gene annotations were queried from Ensembl and UniProt using biomaRt (*83*). Gene set enrichment analysis was carried out using ClusterProfiler (*84*) with an adjusted p-value cut-off of <0.05 and minimum gene-set size of 3.

Solid phase extraction of *N*-acylamides from mouse cecal and human fecal samples

Fifty milligrams of each frozen human fecal sample or 100 milligrams of each frozen mouse cecal sample were weighed into a 2 mL polypropylene snap-cap vial. Nine hundred microliters of ice-cold acetonitrile/methanol [99:1 (v/v)] was added to precipitate proteins. The vial was then vortexed for 30 seconds followed by 10 minutes of sonication on ice. After vortexing again for 10 seconds, the entire homogenate was transferred into a Bond Elut Lipid Extraction 1 mL cartridge (Agilent). Cartridges were placed on a 12-port Visiprep™ SPE Vacuum Manifold. Low pressure (4 psi) was applied to facilitate binding to the column. Once the liquid on the column dried, two 1 mL washes of ACN/water (9:1, v/v) were applied to the cartridges under 4 psi pressure. The cartridge was then dried with higher pressure (6-9 psi). Two 1 mL elutions with chloroform/MeOH (1:1, v/v) were performed under low pressure (1 psi). The combined eluent was evaporated (LabConco CentriVap). The dried residue was reconstituted in 100 µL of n-BuOH/MeOH (1:1, v/v), vortexed for 2 minutes and sonicated for 10 minutes at room temperature. The material was subsequently transferred to a 2 mL sample vial with a glass insert prior to injection into the UHPLC-Qtof-MS instrument for measurement of NAAAs according to the procedure described above.

Phylogenetic analysis of *F. prausnitzii* FAAH orthologs

Sequence similarity (BLASTP) searches were conducted using CGOBPECO_01956 as a query and strict thresholds (Score ≥ 400, Identity ≥ 50%, e-value ≤ 10^{-6}). A multiple sequence alignment of the resulting 218 proteins was generated using MUSCLE (*85*) and a phylogenetic tree was constructed using pairwise distances and the neighbor joining method (*86*). Node repeatability in the tree was assessed via 100 bootstrap replicates (*87*), and the tree was visualized using the plot.phylo function implemented in the ape package in R (*88*).

Supplementary Figures

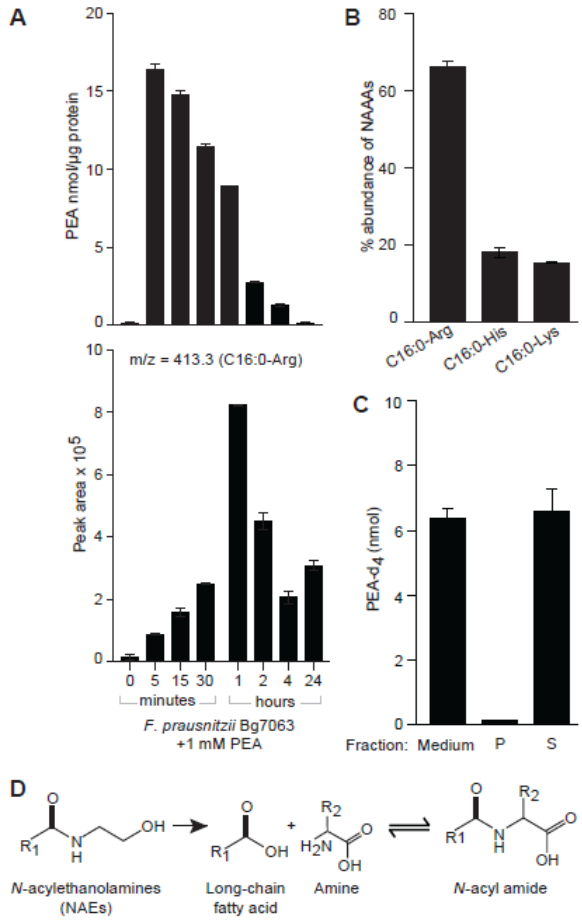


Figure S1. Hydrolysis of PEA by *F. prausnitzii* Bg7063 *in vitro*. (**A**) Time course showing the disappearance of PEA (top subpanel) and the appearance of a PEA metabolite (m/z = 413.3) (bottom subpanel) in monocultures of the bacterium. (**B**) Relative abundance of *N*-palmitoyllysine (C16:0-Lys), *N*-palmitoylhistidine (C16:0-His), and *N*-palmitoylarginine (C16:0-Arg) in *F. prausnitzii* Bg7063 monocultures supplemented with PEA. (**C**) Quantification of PEA-d_4 levels in fractions obtained from sonicates of *F. prausnitzii* Bg7063 monocultures. Abbreviations: P, Pellet; S, Supernatant. (**D**) A proposed two-step mechanism for the enzyme's hydrolysis of *N*-acylethanolamines and reversible hydrolysis and synthesis of *N*-acylamides. Mean values ± SD are plotted in panels A-C.

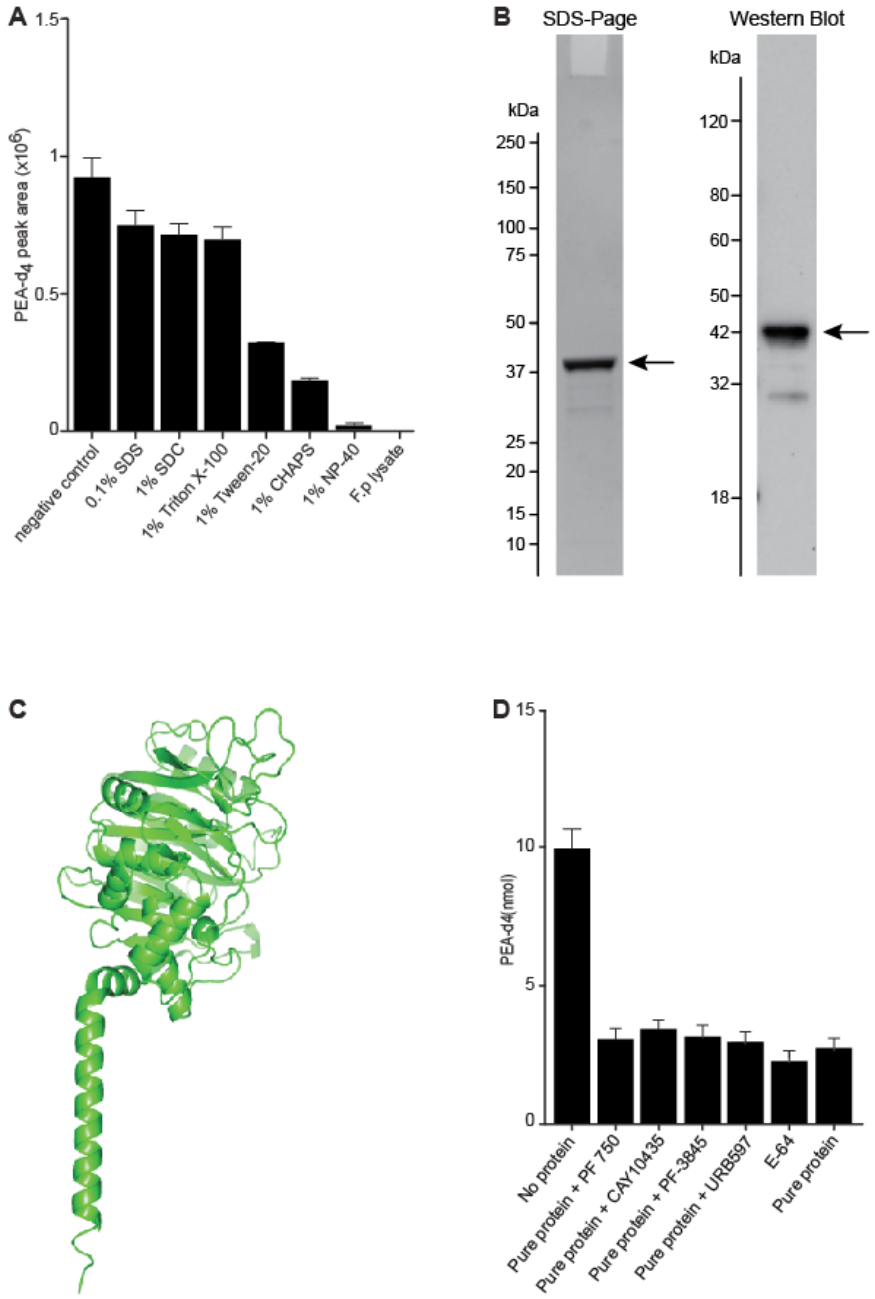
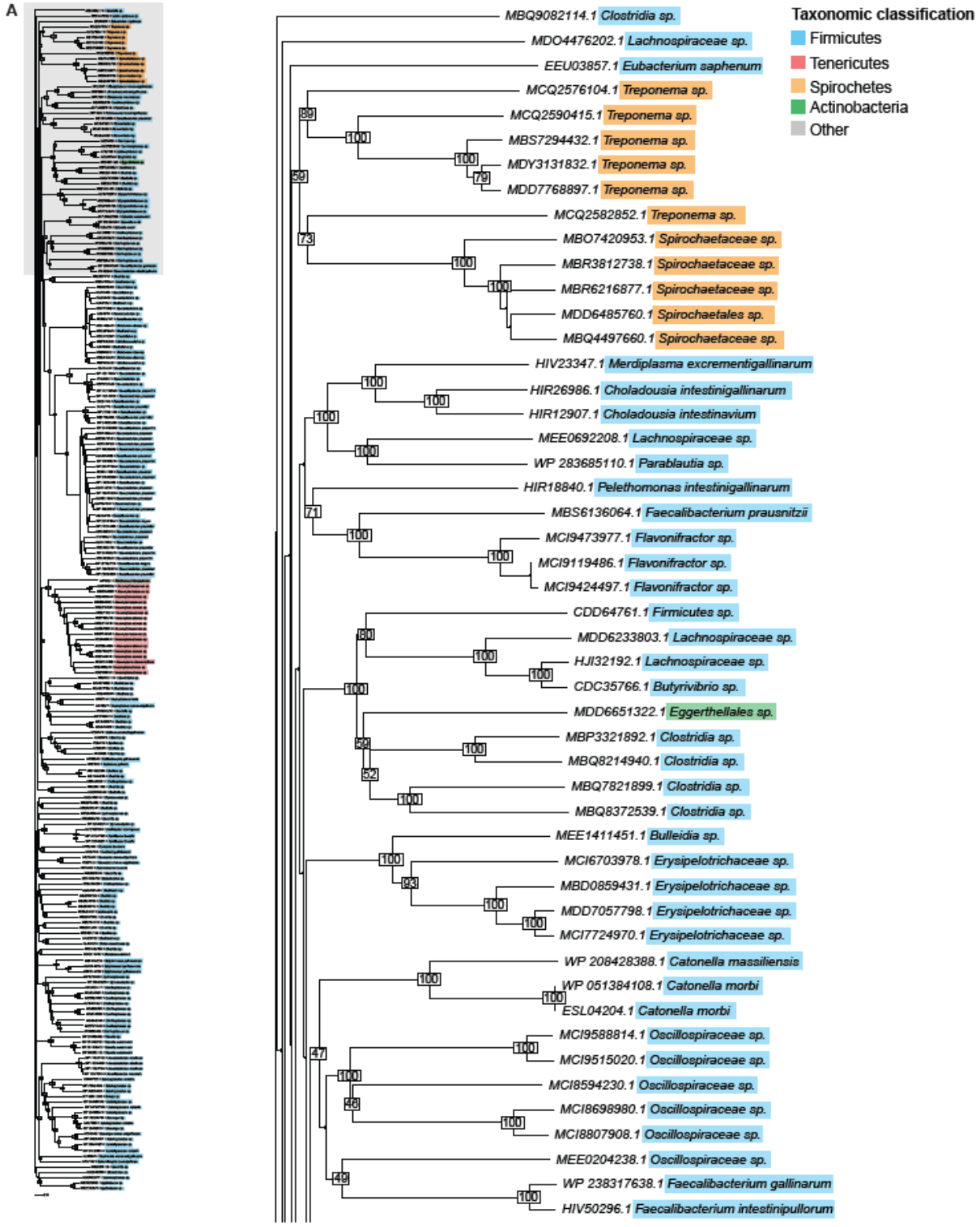


Figure S2. Purification of *F. prausnitzii* FAAH. (**A**) Quantification of PEA-d_4 levels in reaction mixtures incubated for 1h with indicated detergent extracts of cell pellets generated from a 500 µL monoculture of *F. prausnitzii* Bg7063 (n=3 replicates/condition). Mean values ± SD are plotted. (**B**) SDS-PAGE and Western blot analyses of a His-tagged, signal sequence deficient *F. prausnitzii* FAAH protein expressed in and purified from *E. coli*. The smaller band represents a proteolytic product lacking the 19 N-terminal and 23 C-terminal amino acids of the enzyme. (**C**) 3D structure of *F. prausnitzii* FAAH predicted by Alphafold2. (**D**) PEA-d_4 levels in reaction mixtures treated with the indicated inhibitors at 2.5 µM. (n=3 replicates; mean values ± SD shown).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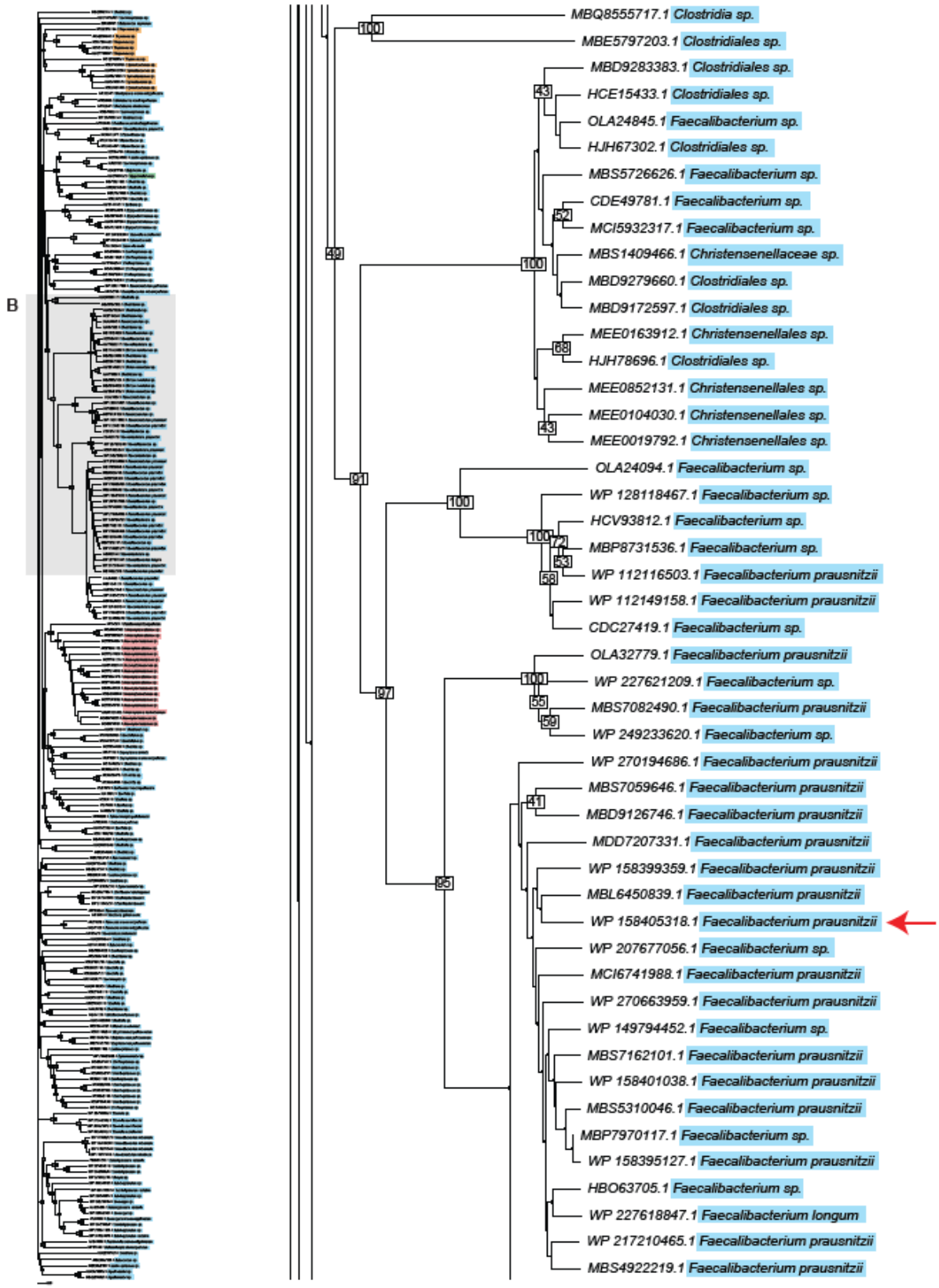
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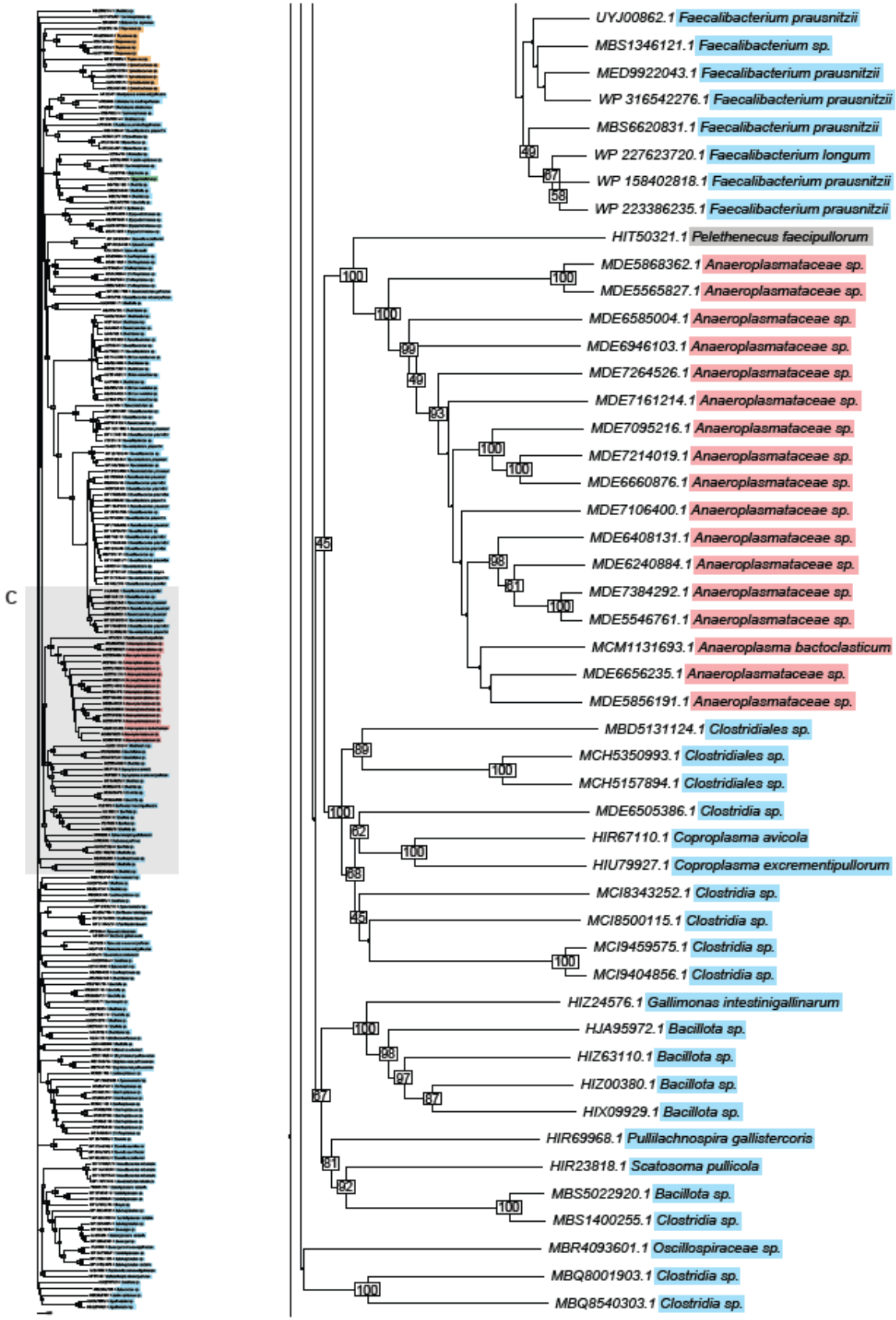
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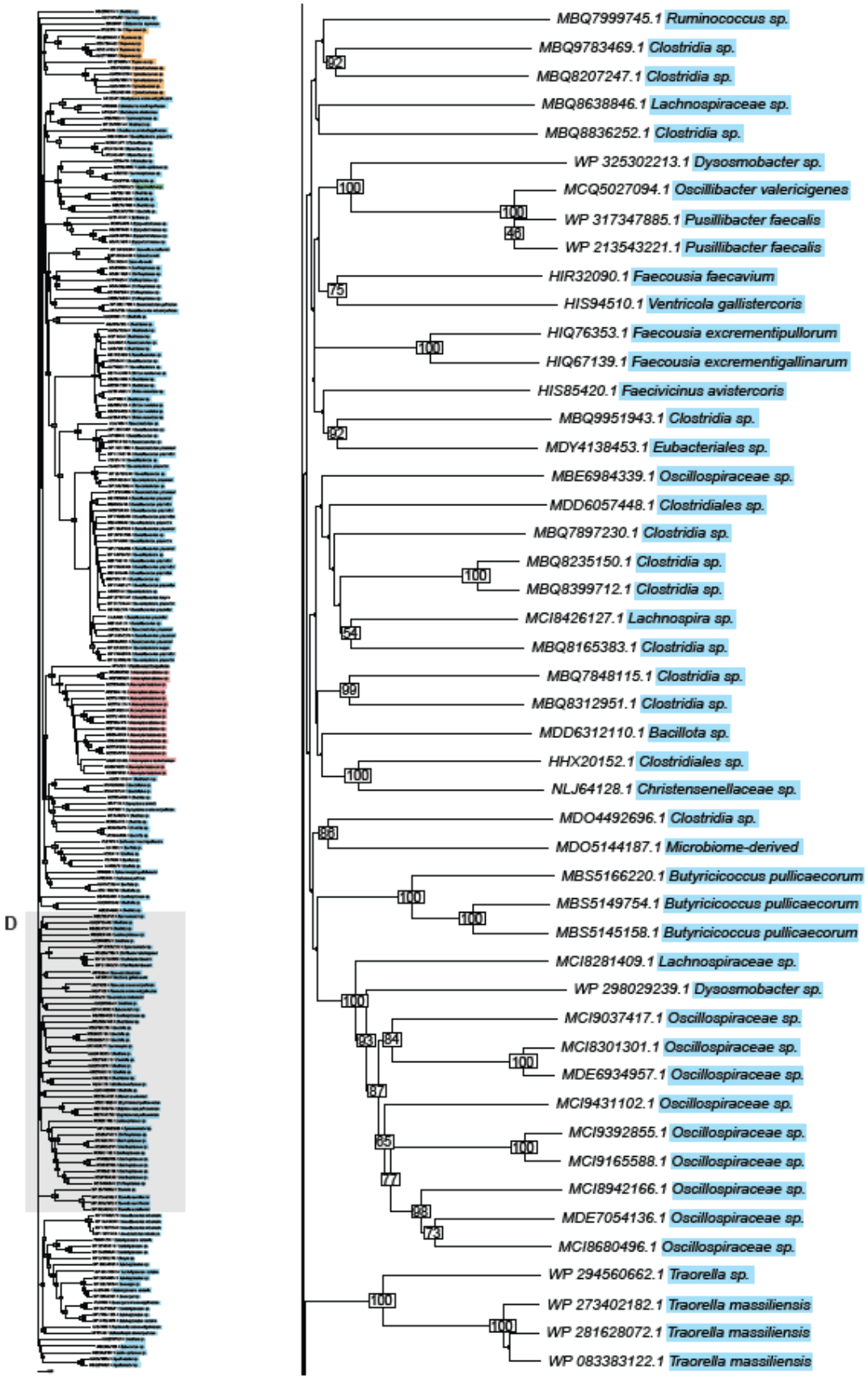
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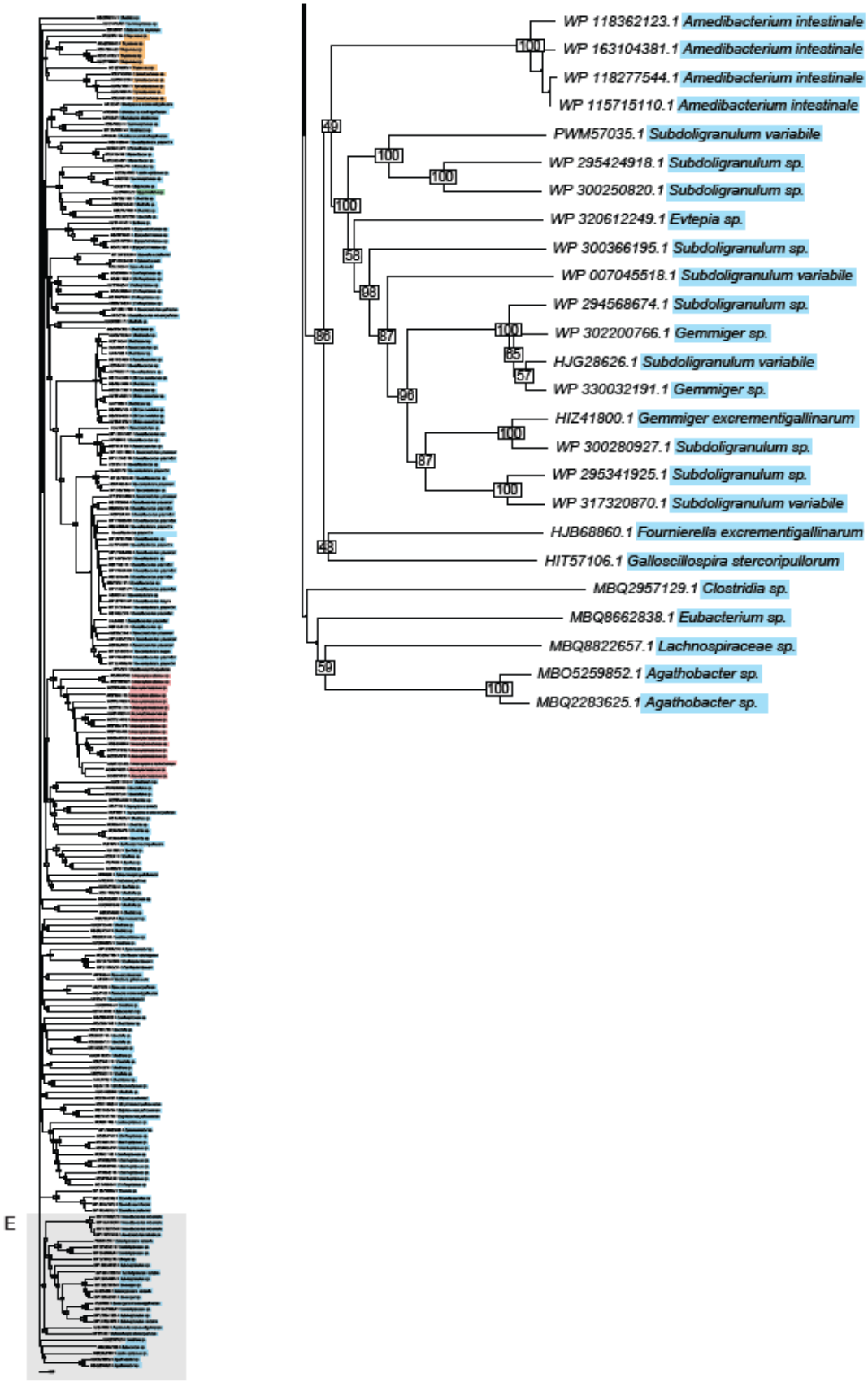
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Figure S3. Phylogenetic analysis of 218 protein sequences that bear sequence similarity to *F. prausnitzii* FAAH. Tree depicting the relationship between the 218 protein sequences that were identified by BLASTP similarity searching using *CGOBPECO_01956* as a query. The numbers at the nodes are bootstrap percentages and indicate the confidence levels of the cluster descending from that node across 100 replicates. Only bootstrap percentages ≥ 40 are shown. *F. prausnitzii* Bg7063 (accession number WP 158405318.1) is indicated with a red arrow. To accommodate the size of the phylogenetic tree, the figure is split into five pages. The grey shaded area (with letters A-E) on the left of each page corresponds to the magnified portion of the tree on the right side of each page.

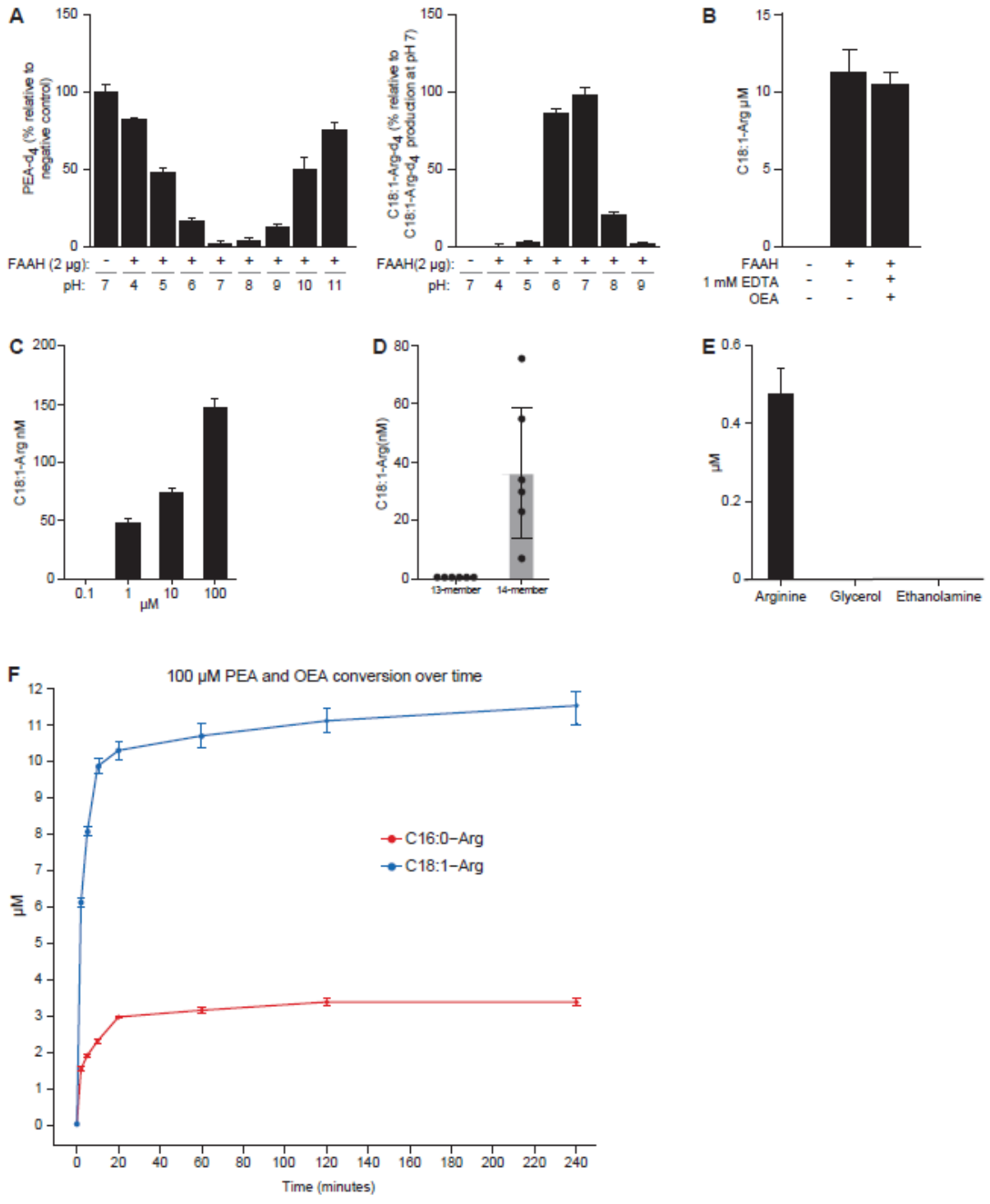

Figure S4. Characterization of purified *F. prausnitzii* FAAH. (**A**) pH sensitivity of *F. prausnitzii* FAAH catalyzed hydrolytic and synthetic activities. (**B**) The formation of C18:1-Arg when 2 µg of purified *F. prausnitzii* FAAH protein was incubated for 1 h with 100 µM OEA and 100mM arginine in the presence and absence of 1 mM EDTA. (**C**) Quantification of C18:1-Arg in a reaction mixture containing 2 µg purified *F. prausnitzii* FAAH protein, 100 µM oleic acid

and 100 mM arginine at the indicated concentration after a 1 h incubation. (**D**) Quantification of C18:1-Arg levels in cecal samples harvested from mice colonized with either the 14-member or the 13-member bacterial consortium. (**E**) Levels of arachidonylarginine, 2-arachidonylglycerol and anandamide in reaction mixtures containing 2 μg of purified *F. prausnitzii* FAAH protein, 100 μM of arachidonic acid and 100 mM of either arginine, glycerol or ethanolamine, respectively. Triplicate reactions were incubated for 1h. Mean values ± SD are plotted. (**F**) Quantification of C16:0-Arg and C18:1-Arg in triplicate reaction mixtures incubated for indicated times, each containing 2 μg of purified *F. prausnitzii* FAAH protein, 100 μM PEA or 100 μM OEA and 100mM arginine. Mean values ± SD are plotted.

Other Supplementary Data (separate .xlsx file)

Supplementary tables S1-S11

Table S1: Cecal analytes (features) identified by untargeted LC-Qtof-MS.

Table S2. Effects of FAAH inhibitors. (**A**) NAEs in feces collected from mice treated with a pharmacologic inhibitor of host *N*-acyl phosphatidylethanolamine-specific phospholipase D (LEI-401). (**B**) PEA-d_4 levels in *F. prausnitzii* Bg7063 monocultures treated with human FAAH inhibitors, PF-3845 and URB597.

Table S3: Identification of the *F. prausnitzii* Bg7063 FAAH enzyme. (**A**) Candidate *F. prausnitzii* genes with potential *N*-acylamide hydrolase activity inferred after sequencing tryptic peptides generated from proteins in the most enzymatically active fraction recovered after ion-exchange chromatography. (**B**) Sequence of *CGOBPECO_01956* encoded protein. (**C**) Orthologs of the *F. prausnitzii* FAAH gene *CGOBPECO_01956* in the fully sequenced genomes of *F. prausnitzii* deposited in the NCBI genome database (updated April 9, 2024).

Table S4: Phylogenetic analysis of *F. prausnitzii* FAAH orthologs. List of orthologs that satisfy strict BLASTP thresholds (Score≥400, Identity≥50%, e-value≤10^{-6}) in the NCBI non-redundant database (updated May 2, 2024).

Table S5: Fatty acid, amine, *N*-acyl amide and *N*-acyl homoserine lactones substrates used for *in vitro* enzymatic assays. (**A**) Fatty acid panel. (**B**) Amine panel. (**A**) *N*-acyl amide panel. (**A**) *N*-acyl homoserine lactones panel.

Table S6: Percent agonist activity of three NAAAs (C18:1-Arg, C18:1-His, and C16:0-Arg) against a DiscoverX panel of 73 GPCRs and 18 nuclear receptors. Receptors that are activated above a threshold value (50%) by the indicated metabolite are highlighted in yellow.

Table S7. Differentially expressed genes in tissues of germ-free mice treated with C18:1-Arg or C18:1-His. (**A**) Ileum. (**B**) Jejunum. (**C**) Colon. (**D**) Liver.

Table S8. Gene set enrichment analysis of RNA-Seq datasets generated from the intestine and liver of germ-free mice treated with C18:1-Arg or C18:1-His. (**A**) Ileum. (**B**) Jejunum. (**C**) Gene Ontology Biological Pathways that were significantly enriched in differentially expressed genes.

Table S9. Result of blastp-based sequence analysis of FAAH in *F. prausnitzii* MAGs identified in Bangladeshi children with MAM who participated in the randomized controlled trial of MDCF-2 and RUSF.

Table S10. Abundance of MAG Bg0005, *F. prausnitzii* FAAH transcripts, and OEA in the feces of children treated with MDCF-2.

Table S11. Levels of MAG Bg0005 and NAAAs in the feces of children assigned to the 'low' and 'high' MAG Bg0005 groups in Fig 4E.