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Supplementary Materials for

An engineered *E. coli* Nissle improves hyperammonemia and survival in mice and shows dose-dependent exposure in healthy humans

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Published 16 January 2019, *Sci. Transl. Med.* **11**, eaau7975 (2019)
DOI: 10.1126/scitranslmed.aau7975

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Table S1 (Microsoft Excel format). Differential gene expression comparisons of SYNB1020 and $EcN^{\Delta thyA}$ to EcN in vitro.

Materials and Methods

Bacterial strains and characterization:

EcN was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ E. coli DSM 6601). The genome of the EcN strain was sequenced by Next Generation Sequencing and used as a reference (Genewiz). A series of control EcN strains were engineered to demonstrate effects of specific features of the engineered strains (Table 1, Fig. 1).

The endogenous argR gene was deleted in EcN by an external contract research organization (enEvolv, Boston, MA) using λ-red technology (*81,82*). The *thyA* gene was then deleted in the Δ*argR* strain via λ-red recombination (*81,82*) of a DNA segment containing a chloramphenicol-resistance cassette in the *thyA* gene in a manner that replaced most of the *thyA* coding region with the drug resistance marker. Resistant colonies were selected on LB agar containing chloramphenicol (30 µg/mL) and thymidine (3 mM). Correct recombination events were verified by PCR (primers described in table S12). and further confirmed by lack of growth on LB agar lacking thymidine. The chloramphenicol resistance gene was then excised with the plasmid pCP20 (*81,82*), carrying the gene encoding the flippase-derived recombinase, and pCP20 was subsequently cured by growing cells at 37°C. The gene *argA215* (*argA*^{fbr}) (*36*), (accession #AF008117.1), encoding a feedback-resistant variant of the ArgA enzyme, was synthesized as a DNA segment in which the coding region for *argA215* was fused to the anaerobic-inducible promoter P_{fnrS} (Genewiz; (*7,47–49*)) and integrated into the intergenic region separating the *malE* and *malK* loci via λ-red recombination of a fragment containing P_{fnrS}-*argA*^{fbr} flanked by ~1kb of homology to *malE* and *malK* on either end (table S12)

All insertions and deletions were confirmed by Sanger sequencing of PCR products spanning the desired locus. Strain genotypes are described in Table 1. Analytical methods for quantification of ammonia and arginine are found in the supplementary materials and methods.

Growth and induction of strains in fermenters

A frozen aliquot from a strain cell bank was thawed and inoculated in 50 mL of FM1 medium (Table S13) in a 500 mL Ultra-Yield flask (Thomson). Cells were grown at 37°C with shaking at 350 rpm until an optical density $(OD)_{600}$ of >10 was reached, at which point a portion of the culture was transferred to 4 L of FM1 in an Eppendorf BioFlow 115 bioreactor (starting OD_{600} of ~0.02). The fermenter was controlled at 50% dissolved oxygen with agitation, air, and oxygen supplementation, and controlled to pH 7 using ammonium hydroxide. At OD_{600} of 3-5, the P_{fnrS} promoter controlling the *argA215* gene was activated by creation of a low oxygen environment (10% dissolved oxygen), thus inducing the arginine biosynthetic pathway. Cells were harvested by centrifugation at 4500×g for 30 min at 4°C, resuspended in formulation buffer (15% v/v glycerol, 5% w/v trehalose, and 10 mM 3- [N morpholino] propanesulfonic acid buffer [MOPS], pH 7.3), and stored at -80°C until the day of testing. OD_{600} was measured on a BioPhotometer Plus spectrophotometer (Eppendorf).

Analysis of EcN and derivative strains by flow cytometry

EcN or EcN-GFP (green fluorescent protein-labeled) were grown overnight in LB at 37°C with shaking (250 rpm). Overnight cultures were used to inoculate 500 mL of LB in 2L baffled flasks (1:100 back-dilution from overnight cultures). Cultures were grown shaking (250 rpm) at 37°C to maintain the aerobic state. At an OD_{600} of ~1, an aliquot of EcN-GFP was moved to an

anaerobic chamber (Coy) for 4 hours as a control for activation of the P_{fnrS} promoter (fig. S1). The remainder of the culture remained aerobic and was grown until an OD_{600} of ~5. Cells were harvested by centrifugation and washed once with ice-cold PBS. The cells were then resuspended in PBS containing 15% glycerol and stored at -80°C. A 1×10^{10} dose of EcN-GFP was used to orally dose C57BL/6 mice. Fecal samples were collected before or 3 or 6 hours after oral dosing, resuspended in PBS, homogenized for 30 seconds, and kept on ice before analysis on flow cytometer. A Macsquant VYB analyzer was used for sample analysis. A blue (488nm) laser was used for excitation and the B1 channel (525/50) was used for data collection and GFP signal measurement. Since EcN cells are relatively small for measurement by flow cytometry, proper setting of Forward Scatter (FSC) and Side Scatter (SSC) for Nissle cells also displays small particles and bacteria from homogenized feces; however, a clear GFP signal can be distinguished from this background. Data were plotted by having SSC on the y-axis and GFP signal on the x-axis.

Bacterial RNAseq analysis

To characterize the transcriptional profile of EcN Str^R, $EcN^{\Delta thyA}$, and SYNB1020, triplicate cultures of each strain were incubated overnight in LB broth supplemented with 10 mM thymidine at 37°C in an orbital shaker at 250 rpm. Overnight cultures were then diluted 100-fold in 3 mL LB broth supplemented with 10 mM thymidine and incubated for 2 hours at 37°C in an orbital shaker set to 250 rpm. These subcultures were then incubated an additional 2 hours at 37°C without shaking to induce gene expression under microaerobic conditions. Each subculture was then centrifuged at 3220×g for 5 mins to pellet cells, supernatants were removed, and cell pellets were frozen on dry ice. To extract RNA, frozen cell pellets were resuspended in 400 µL

cold Trizol reagent (Thermo Fisher) and incubated at room temperature for 5 mins, followed by addition of 160 μL chloroform. Samples were mixed by inversion, incubated at room temperature for 2 mins, and centrifuged for 15 mins at 12,000×g at 4°C. 200 μL of the upper, aqueous phase was then transferred to a new tube. RNA was precipitated by the addition of 200 μL isopropanol and incubation at room temperature for 10 mins, prior to centrifugation at 12,000×g for 10 mins at 4°C. The supernatants were discarded, and pellets were washed with 400 μL 75% ethanol (prepared with nuclease free water) and centrifuged at 7500×g for 5 mins at 4°C. Supernatants were then discarded, and the resulting pellets were air dried for 15 mins and resuspended in 20 μL nuclease-free water. Genomic DNA was digested using DNAse I (Sigma AMPD1), following the manufacturer's instructions. Ribosomal RNA depletion and cDNA synthesis were performed as previously described (*83*). Sequencing libraries were prepared for each cDNA using the Nextera XT DNA library preparation kit (FC-131-1024; Illumina). Nextera XT libraries were sequenced in multiplex using the MiSeq platform using the MiSeq v3 150 reagent kit (MS-102-3001; Illumina) to obtain 150 bp unpaired reads. Sequencing reads were demultiplexed using the BaseSpace FASTQ generation workflow (Illumina). Demultiplexed reads were then trimmed using Trimmomatic with the following options: ILLUMINACLIP: TruSeq3-SE.fa:30:10 LEADING:20 TRAILING:20 SLIDINGWINDOW:4:25 MINLEN:50 (*84*). Trimmed reads were then aligned to the EcN complete genome (*85)*; Genbank accession CP007799.1) using bowtie1 version 1.2.1.1 using the following options: -v 2 -a --best --strata --sam --threads 4 (*86*). Feature counts (number of reads per Nissle gene) were obtained using HTSeq Count using the following options: -mode=union --stranded=no (*87*). Finally, pairwise differential expression testing was conducted using DESeq2 (*53*). The set of differentially expressed genes was mapped to metabolic pathways using KEGG orthology annotations (*88*).

In vitro ammonia consumption assay

Bacterial cells were grown at 37°C, 250 rpm overnight in LB supplemented with 10 mM thymidine. The bacteria were then diluted 100-fold in 20 mL LB with 10 mM thymidine and incubated at 37°C, 250 rpm aerobically. After 2 hours, the cultures were placed in static conditions for activation of the P_{fnrS} promoter and incubated for an additional 2 hours. The cells were then spun down at 3200×g and resuspended in 10 mL M9 minimal media without NH_4Cl and containing 0.5% glucose. The cell concentrations of the bacterial suspensions were determined on a cell counter (Cellometer X2, Nexcelom) using 1,000-fold dilutions prepared in phosphate buffered saline (PBS). The concentrations of the suspensions were adjusted to 10^9 cells/mL solution with M9 media without NH_4Cl, supplemented with 0.5% glucose and 10 µg/mL chloramphenicol. Chloramphenicol was used to limit subsequent consumption of ammonia due to bacterial division or de novo protein synthesis during the assay. The concentration of chloramphenicol used in these assays was 2.5-times the minimum inhibitory concentration (MIC) of 4 µg/mL, which was determined by broth microdilution susceptibility testing by Jones Microbiology Institute (JMI Inc). Cell suspensions were pipetted into a 96 deep well round-bottom polypropylene plate in triplicate, 1 mL per well. Each well was spiked with 10 µL of 0.5 M NH_4Cl prepared in deionized water to a final 5 mM concentration to initiate the reaction. The plate was then mixed, covered with a Breathe-Easier membrane (Sigma) and incubated for 3 hours at 37°C. Samples (150 µL) were taken at 0, 1, 2, 3 hours, placed on dry ice and stored at -20°C until NH_3 analysis, with NH_3 quantified using the indophenol blue method as follows. First, samples were thawed on ice and centrifuged at 3200×g for 5 minutes and 0.5, 1, 2, 3, 4, and 5 mM NH_4Cl standards were prepared in M9 without NH_4Cl, 0.5% glucose. 50 µL of

each sample and standards was then mixed with 100 μL of 0.33 M phenol, 0.32 M NaOH, 50 μL of 0.02% sodium nitroprusside, and 100 μL of sodium hypochlorite in a 96-well flat-bottom polystyrene plate. After mixing by pipetting, the plate was placed at 37°C for 15 minutes and the absorbance read at 635 nm.

Mouse Studies

Male OTC *spf*^{ash} and female Balb/cJ (Jackson Laboratory) mice were housed (5 per cage) in Innocage cages (Innovive) on ventilated racks, maintained at 21 ± 1°C, 40-70% relative humidity and allowed to acclimate for a minimum of 4 days prior to initiation of studies. Mice were fed Picolab Diet #5053 chow (Purina) and water *ad libitum* unless otherwise indicated. For all studies, bacteria were administered during the light phase of the daily light/dark cycle, which did not coincide with feeding time.

Breeding pairs for the OTC *spf*^{ash} mice were obtained from The Jackson Laboratory. Colonies of *spf*^{ash} mice were generated at Charles River Labs and transferred to our facility. For the first study (Fig. 2A), 5- to 10-week-old male OTC *spf*^{ash} mice were switched to a 70% high-protein diet (TD.150582; ENVIGO) for approximately 36 hours prior to the first oral administration of vehicle (15% glycerol in PBS), autoclave-killed SYNB1020 at 1×10^{10} CFU, or SYNB1020 at 1×10^{9}, 5×10^{9}, or 1×10^{10} CFU. Animals remained on high-protein diet for an additional 12 hours and then received a single dose of vehicle (water) or peptone (50 mg/mouse, Peptic Digest Sigma-Aldrich]), immediately followed by a second oral dose of vehicle or bacteria. Two hours after administration of vehicle or bacteria, blood ammonia levels were determined using a Blood Ammonia Analyzer (Pocket ChemTM BA Blood Ammonia Analyzer, PA-4140—Arkray Global

Business Inc.). Mice survival was monitored for 24 hours following administration of the last dose of bacteria.

For the study shown in Fig. 2B, 5 to 12-week-old male OTC *spf*^{ash} mice were used. Mice were administered either vehicle (H_2O), EcN$^{\Delta argR \Delta thyA}$ or SYNB1020^{KR} by oral gavage BID and switched to 70% high-protein diet (TD.150582; ENVIGO) after the second of the BID doses on Day 3. Mice receiving high-protein diet were maintained on this diet until study end (Day 9). The mice in normal chow were maintained on this diet throughout the study. On Day 5 of the study (Day 2 post change to high-protein diet), all mice were administered a single oral dose of vehicle, EcN$^{\Delta argR \Delta thyA}$ or SYNB1020^{KR}. Four hours later, blood ammonia concentrations were assessed as described above. Survival was monitored while mice continued to be dosed BID until study end (Day 9).

For Fig. 2C, nine-week-old Balb/cJ mice were maintained on regular chow and concomitantly administered thioacetamide (TAA) at 150 mg/kg intraperitoneally (IP) 3 times per week with daily vehicle (15% glycerol in PBS) or bacteria (EcN or SYNB1020 at 1×10^{11} CFU, PO, BID) based on published methods (*89*). Following 3 weeks of treatment, blood ammonia was determined as described above.

For the studies shown in table S2, the cecolonic contents of each mouse were diluted to 1 mL with PBS. Ten-fold diluted doses of SYNB1020^{KR} were added to each tube to create a dose

range from 10^{10} CFU per tube to 10^7 CFU per tube. The samples were incubated at 37 °C for 68 hours with shaking, and aliquots were removed periodically, serially diluted, and plated on selective media in order to determine the number of viable CFU at each time point. For the studies shown in tables S3 and S4, female C57BL6 were administered three oral doses, twice daily (except Day 3, when mice only received 1 dose) of SYNB1020^{KR} or EcN^{StrR} (1×10^{10} CFU) on three consecutive days. Mice were euthanized four hours after the final dose and the contents of their cecum and colon were collected by first removing the tissue and scraping the contents into tubes, followed by flushing with 1 mL of cold, sterile PBS and collecting the effluents into the same tubes as the scraped material. To enumerate the number of bacteria that survived the transit though the upper GI tract to the cecum and colon, the samples collected from the mice were serially diluted (1:10), plated on LB agar plates containing selective antibiotics (SYNB1020^{KR}-kanamycin, 100 μg/mL, thymidine 3 mM; EcN^{StrR}-100 μg/mL streptomycin), and incubated overnight at 37°C. The next day, colonies were counted to determine the number of CFU present in the samples (table S3). To determine the rate of arginine production by SYNB1020^{KR} compared to EcN^{StrR} bacteria that had survived transit to the cecum/colon compartment, 100 μL of the samples collected from the mice above were diluted in 2 mL of arginine assay media (M9 Minimal Medium supplemented with 0.5% glucose, 10 mM thymidine, 18 mM ^{15}N-NH_4Cl), incubated at 37°C with shaking for three hours and then the samples were analyzed by LC-MS/MS for ^{15}N-arginine (table S4).

28-day repeat-dose toxicology study of SYNB1020 in male and female CD-1 mice

A 28 day repeat dose toxicology study was conducted with SYNB1020 in CD1-mice. Groups of 16 male and female mice were dosed with vehicle alone (PBS/glycerol/sodium bicarbonate) or

SYNB1020 at 2.6×10^{9}, 2.6×10^{10}, or 7.8×10^{10} CFU/dose BID in a volume of 0.3 mL/dose. Ten mice/sex/group were designated for terminal necropsy at Day 29, and additional groups of 6 mice/sex/group were followed for a 2-week post-dose recovery period. Fecal samples were collected weekly from the vehicle control, low dose (2.6×10^{9} CFU/dose), or high dose (7.8×10^{10} CFU/dose) mice during the dosing period and during the recovery period on Days 30, 35, and 40, for the presence of SYNB1020 in the stool using a validated qPCR assay. Upon completion of dosing or following the 2-week recovery period, surviving mice were sacrificed, full gross necropsies were performed, tissues were collected for histological examination. Samples of key organs, including the GI tract (stomach, ileum, duodenum, jejunum, and colon), liver, spleen, urinary bladder, and gonads, were also collected during the terminal necropsy (Day 29) and assessed for the presence of SYNB1020 DNA using the same qPCR method.

Analytical methods

L-Arginine was quantified using a triple quadrupole liquid chromatography-mass spectrometry (LC-MS) Thermo TSQ Quantum Access Max system. Standards were prepared in water with the following concentrations: 0.8, 4, 20, 100, 250, 500, and 1000 µg/mL. Samples were stored at -80°C prior to analysis. 20 µL of the standards and thawed samples were transferred to a 96-well plate, followed by the addition of 80 µL of 75% acetonitrile, containing 25 µg/mL of $^{13}C_6$,$^{15}N_4$ -L-arginine internal standard. The plate was then sealed and centrifuged at 4000 rpm for 5 min at 4°C. In another V-bottom- plate, 5 µL of the supernatants were mixed with 85 µL 10 mM $NaHCO_3$ pH 9.4 and 10 µL 10 mg/mL dansyl chloride (prepared in acetonitrile). The plate was heat-sealed and mixed using a 96-well plate thermomixer for 5 sec at 400 rpm, followed by incubation at 60°C for 45 min for derivatization. The samples were then centrifuged

at 4000 rpm for 5 min at 4°C. From the previous step, 20 µL of the supernatant was then transferred into a 96-well round-bottom plate and further diluted with 180 µL of 0.1% formic acid in water.

The injection volume used was 10 µL and the run time was 6 min at a flow rate of 0.4 mL/min. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. Chromatographic separation was carried out using a Luna C18(2) column (5 µm, 50×2 mm) at RT with the following gradient: 10%B from 0 to 1 min, 10→90% B from 1 to 3min, 90%B from 3 to 4 min, 10%B from 4 to 6 min. Multiple reaction monitoring (MRM) in positive mode was used for tandem MS analysis. The following mass transitions were monitored for quantitation: L-arg (412.1/170.1) and $^{13}C_6$,$^{15}N_4$ -L-arginine (418.1/170.1).

Quantification of L-arginine was performed using a triple quadrupole LC-MS Thermo TSQ Altis system. Standards were prepared in water with the following concentrations: 0.8, 4, 20, 100, 250, 500, and 1000 µg/mL. Samples were stored at -80°C prior to analysis. In a 96-well plate, 10 µL of the standards and thawed samples were transferred, followed by the addition of 90 µL of L-arginine- $^{13}C_6$,$^{15}N_4$ internal standard solution (5 µg/mL L-arginine- $^{13}C_6$,$^{15}N_4$ dissolved in 70% acetonitrile in water). The samples were then centrifuged at 4000 rpm for 5 min at 4°C, followed by transferring 80 µL of the supernatant into a 96-well round-bottom plate.

The injection volume used was 10 µL and the run time was 13 min at a flow rate of 0.3 mL/min. The mobile phase was 50% methanol with 20 mM ammonium acetate, at 70% isocratic flow. Chromatographic separation was carried out using a Sigma Astec Chirobiotic T column, 5 µm (150×2 mm), set at 40°C. MRM in positive mode was used for tandem MS analysis. The

following mass transitions were monitored for quantitation: L-arg (175.1/70.0), L-arginine-$^{13}C_6$,$^{15}N_4$ (185.0/75.0).

Phase 1 clinical study in healthy human volunteers

Part 1 of the study followed a SAD design with up to 7 dose cohorts receiving SYNB1020 or placebo (3 treated:1 placebo) over 6 days to identify the maximum tolerated dose (MTD) of SYNB1020. Upon determination of the MTD, the study proceeded to enrollment of up to 4 MAD cohorts (6 treated:2 placebo) in Part 2.

Subjects in the first SAD cohort received a single dose of 2×10^9 CFU of SYNB1020 or matching placebo. Sequential cohort enrollment proceeded to escalating pre-specified single dosing day levels, with dose-escalation decisions made based on tolerability, clinical observations, and safety laboratory assessments once the last subject in a cohort was dosed and had at least 72 hours of post-dose observation. A dose-escalation committee comprising the Sponsor's Medical Monitor, the pharmacovigilance physician at the contract research organization (PAREXEL), and the Principal Investigator may have then recommended dose level expansion at the current dose level, escalation to the next higher dose level, or declaration that the MTD had been achieved.

Once a safe dose was established in the SAD part of the study, the first multiple dose cohort was initiated at the MTD dose level or lower, with investigational product (IP) administered TID in MAD Cohorts 1-3. Subjects in each MAD cohort completed 14 days of IP administration and at least 72 hours of post-dose observation prior to being released from the inpatient facility.

The total duration of inpatient monitoring was 6 days in the SAD cohorts and 22 days in the MAD cohorts, during which time subjects underwent baseline evaluations, IP administration, safety monitoring, and collection of blood, urine, and fecal samples for microbial-kinetic and

pharmacodynamic evaluations, including a tracer study for subjects in the MAD part of the study. Subjects were followed for safety and SYNB1020 clearance for up to 100 days following the last study dose.

For the duration of the inpatient stay, subjects received a calorie and protein-controlled diet, as well as a proton pump inhibitor to reduce stomach acid, comprising esomeprazole 40 mg administered once daily (30 to 60 minutes before breakfast). The dosing and study design are shown in fig. S5.

Safety was evaluated by continuous monitoring of adverse event, vital signs, clinical laboratory measurements, ECGs, and physical examinations. Adverse events were coded using the Medical Dictionary for Regulatory Activities (MedDRA), version 20.0. The severity of adverse events and laboratory abnormalities were graded using the National Cancer Institute Common Terminology Criteria for Adverse Events, version 4.03.

The excretion profile of SYNB1020 was assessed in feces from subjects. Feces from each voiding were collected for analysis of SYNB1020 transit through the GI tract. The fecal samples were weighed, homogenized, and a sample was analyzed for SYNB1020 by qPCR. Primer sequences used for these analyses include forward and reverse primers and a probe (TaqMan method described above).

Arginine metabolites from SYNB1020 activity were evaluated in feces, urine and blood from subjects. Feces, urine, and blood samples were collected at screening and on study both before and after administration of SYNB1020 or placebo. The following analytes were measured;

plasma ammonia (24-hour AUC), (blood urea nitrogen), and amino acid analyses, urinary orotic acid, 24-hour nitrogen, and urea, fecal nitrogen, urinary nitrate, urinary total ^{15}N, ^{15}N-urea (N1 and N2), ^{15}N-nitrate, ^{15}N-ammonia, plasma ^{15}N-urea (N1 and N2), ^{15}N-nitrate, and ^{15}N-citrulline. To assess in vivo activity of SYNB1020, an oral $^{15}NH_4Cl$ tracer was administered. Ammonia is endogenously created in the GI tract and it is rapidly converted to other metabolites in the liver. As the pharmacodynamic activity of SYNB1020 is related to ammonia consumption and its conversion into other nitrogenous species (primarily arginine), tracing the transit ammonia required the ability to differentially measure subsequent metabolites of $^{15}NH_4$ in either intermediate (plasma) or final (urinary or fecal) disposition. A urea tracer study was performed in the MAD cohorts using previously published MS methods (*90*).

The ex-vivo activity of SYNB1020 following oral dosing was assessed in feces from subjects. Initially, a standard curve of SYNB1020 activity in naive healthy human stool was prepared. Stool homogenates were homogenized in homogenization buffer (15 mM MOPS, 7.5% trehalose, buffered to pH 7) in a 1:2 (mass: volume) stool:buffer ratio. Following homogenization, 75% glycerol was added in a 1:4 (volume: volume) glycerol:homogenate to obtain a final 15% glycerol concentration before freezing. Based on this homogenization protocol and assuming a fecal density of 1 g/mL, the 50 µL of stool homogenate used in the assay described herein contains 13.3 mg of stool. In 96-well deep well plate, assay reactions were prepared in 950 µL of glucose-containing media with $^{15}NH_4Cl$ as the sole nitrogen source and a 50 µL volume of fecal homogenate (corresponding to 13.3 mg feces). A 13.3 µL volume of cell stocks were pipetted, in duplicate, into appropriate reaction wells to obtain a final 1.33×10^8, 1.33×10^7, 1.33×10^6, and 1.33×10^5 SYNB1020 cells per well, which corresponded to 10^{10}, 10^9,

10^8, and 10^7 cells/g of feces, respectively. The 96-well plates were sealed with a breathable membrane and incubated at 37°C, shaking (850 rpm) using a microplate orbital shaker for 7 hours. Following the 7-hour incubation, the plates were sealed with an aluminum seal and stored at -80°C until $^{15}N_4$-L-arg was quantified by LC-MS/MS.

Supplementary figures and tables

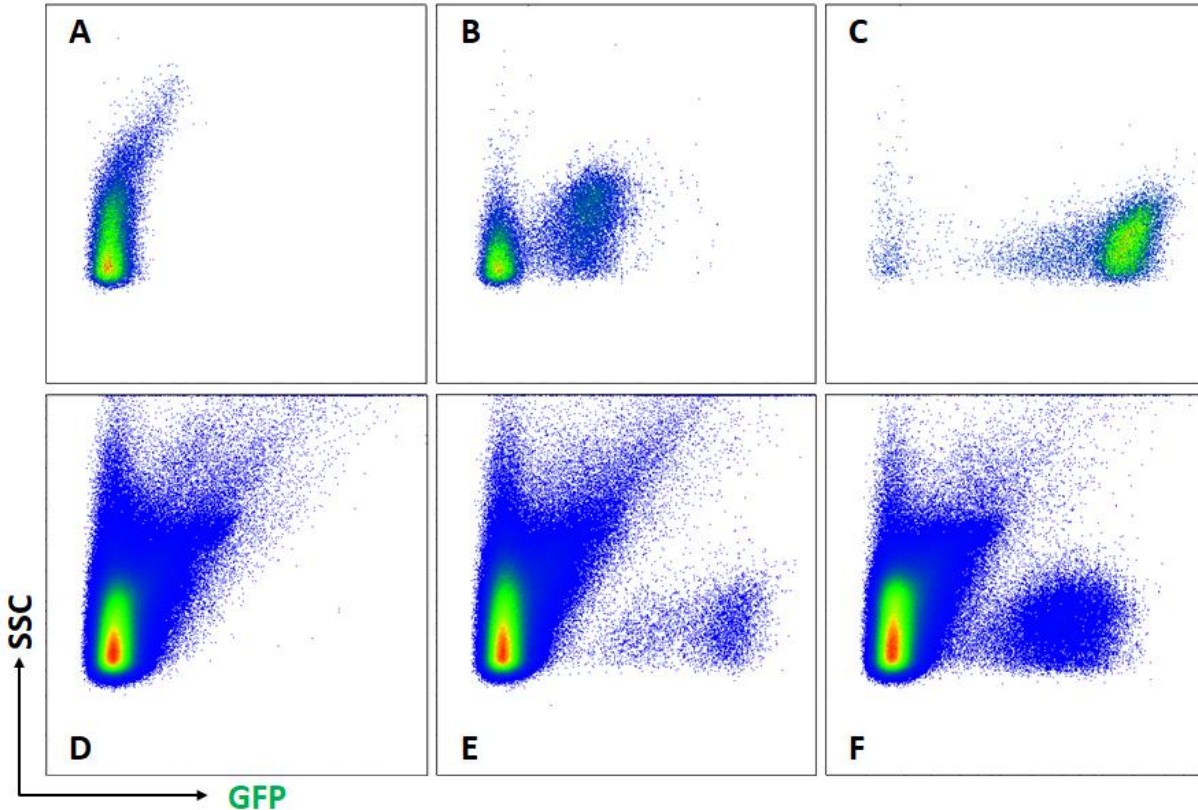

Fig. S1. Demonstration of in vivo induction of the P$_{fnrS}$ using green fluorescent protein as a reporter in EcN strain. EcN and derivative strains analyzed for GFP (green fluorescent protein) fluorescence (x-axis) by flow cytometry in vitro **(A-C)** and in vivo **(D-F)**. **(A)** EcN (control; no *gfp* expression). **(B)** EcN harboring a plasmid containing the *gfp* gene driven by the P$_{fnrS}$ promoter (EcN-GFP) and grown aerobically demonstrates basal activity with a population of cells displaying some GFP fluorescence. **(C)** EcN-GFP grown anaerobically displays high levels of GFP fluorescence, demonstrated by a shift of the population across the x-axis. **(D)** Mouse fecal sample homogenized in PBS and analyzed for GFP fluorescence immediately before oral dosing of EcN-GFP. **(E)** Mouse fecal sample homogenized in PBS and analyzed for GFP fluorescence 3 hours after oral dosing of aerobically grown EcN-GFP. **(F)** Mouse fecal sample homogenized in PBS and analyzed for GFP fluorescence 6 hours after oral dosing of aerobically grown EcN-GFP. A clear GFP-expressing population is observed in mouse feces following dosing with EcN-GFP, demonstrating that the P$_{fnrS}$ promoter is activated in vivo.

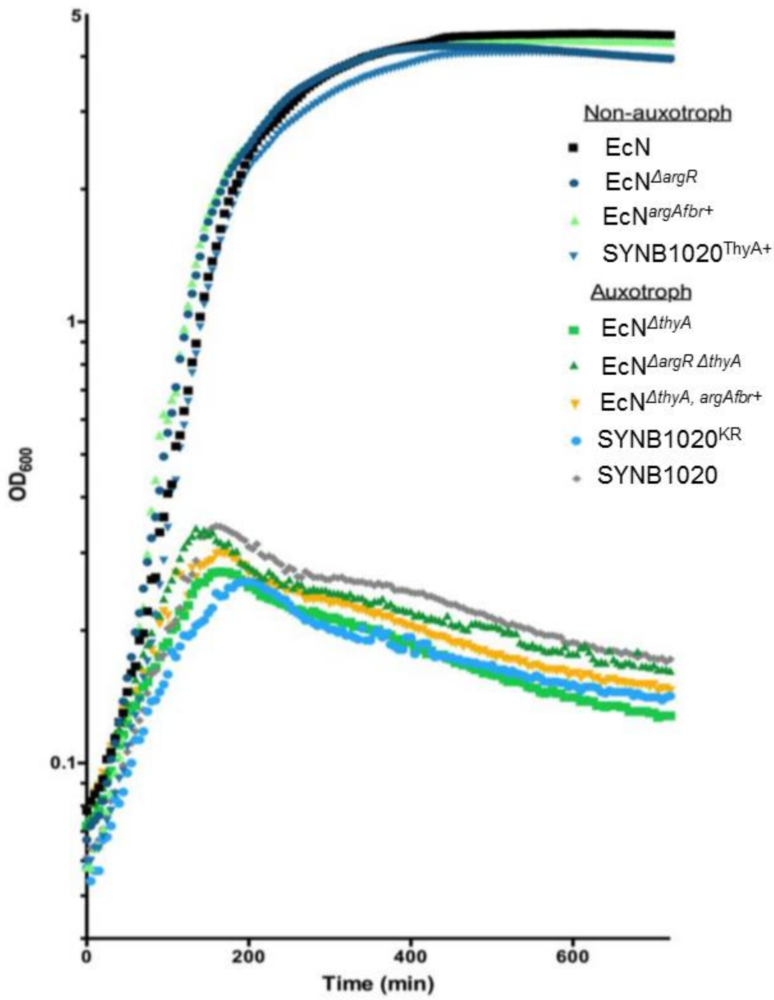

Fig. S2. EcN strains lacking the *thyA* gene fail to grow in rich media lacking exogenous thymidine. Growth curves were performed in a microplate reader in LB media lacking exogenous thymidine. All strains containing a deletion of the *thyA* gene were unable to grow to levels comparable to that of strains with an intact *thyA* gene. The average of three biological replicates is plotted for each time point.

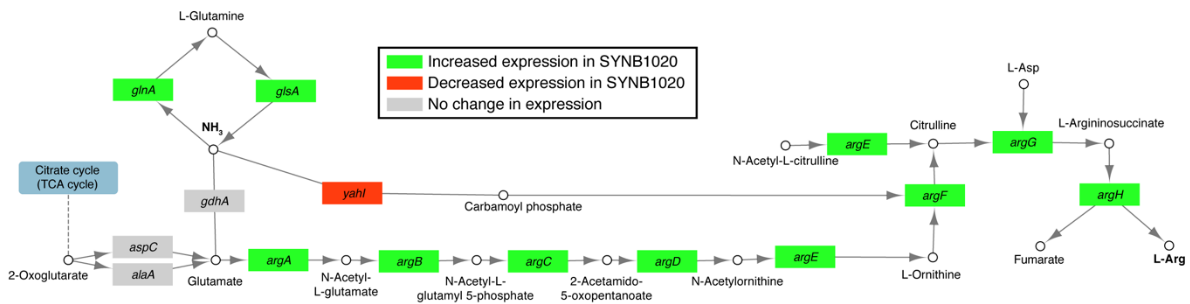

Fig. S3. Differential gene expression for SYNB1020 compared to EcN for genes in the L-arg biosynthesis pathway. Box labels correspond to EcN gene name abbreviations. Green and red boxes indicate genes that show significantly increased and decreased expression, respectively, in SYNB1020 compared to EcN (DESeq2 adjusted p-value < 0.1; see table S1 for differential expression statistics of all EcN genes, including statistical significance).

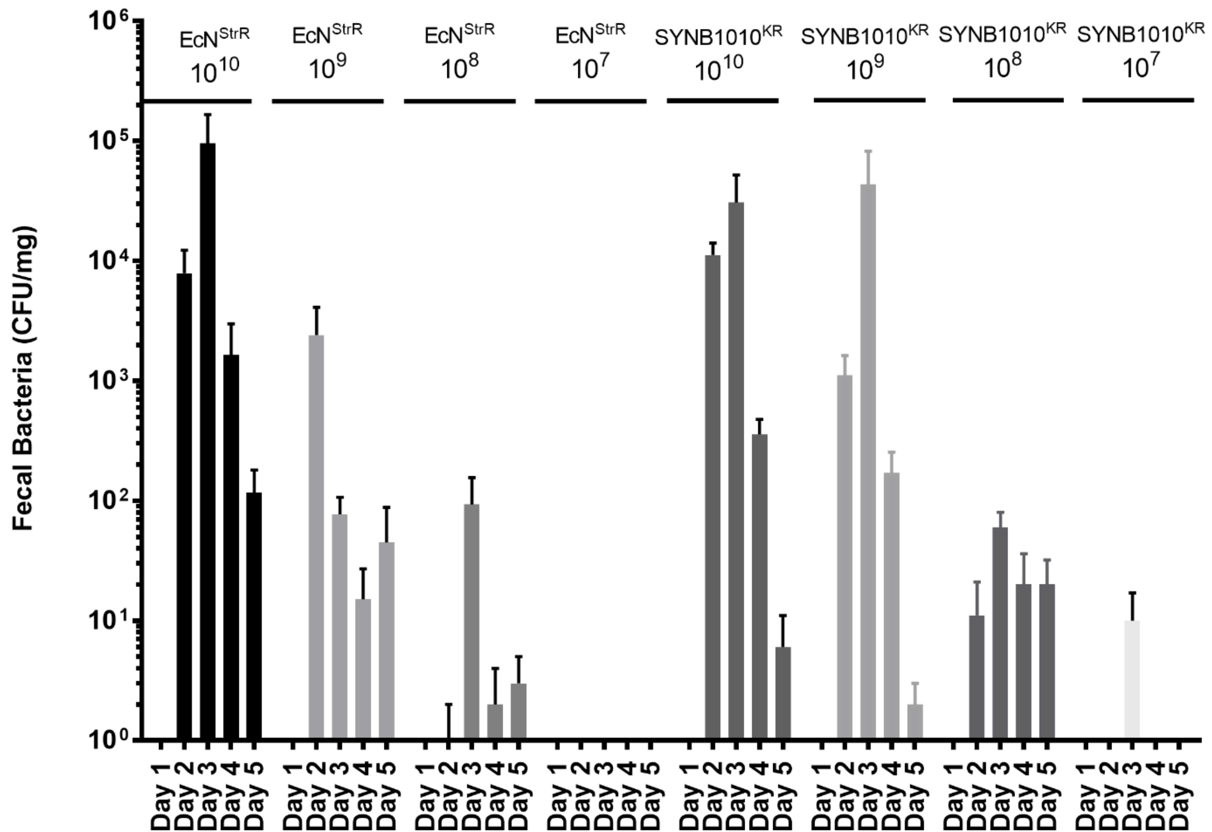

Fig. S4. Dose-dependent fecal excretion of EcN and SYNB1020 strains in mice. Female C57BL/6 mice (n=3/group)were administered increasing dose levels of the control strain EcN^{StrR} (1×10^7, 1×10^8, 1×10^9, and 1×10^{10} CFU/mouse, respectively) or $SYNB1020^{KR}$ (1×10^7, 1×10^8, 1×10^9, and 1×10^{10} CFU/mouse, respectively). Fecal samples from individual mice were collected on Days 1-5 postdosing, then weighed and processed for serial dilution plating to determine viable CFU/mg of feces.

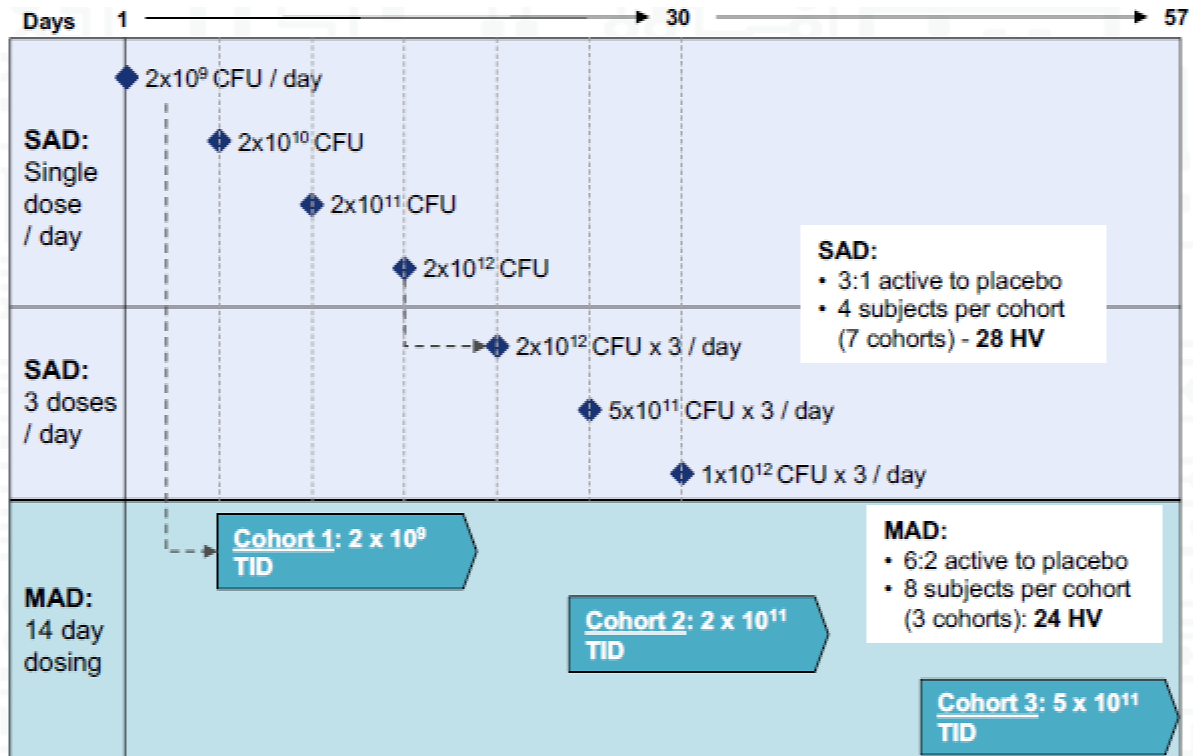

Fig. S5. Study design of a randomized, double-blinded, placebo-controlled study to assess the safety, tolerability, and pharmacodynamics of SYNB1020 in healthy volunteers. Seven single-ascending dose cohorts with 4 subjects per cohort were enrolled, and 3 multiple-ascending dose cohorts of 14 days of dosing with 8 subjects each; total N=52.

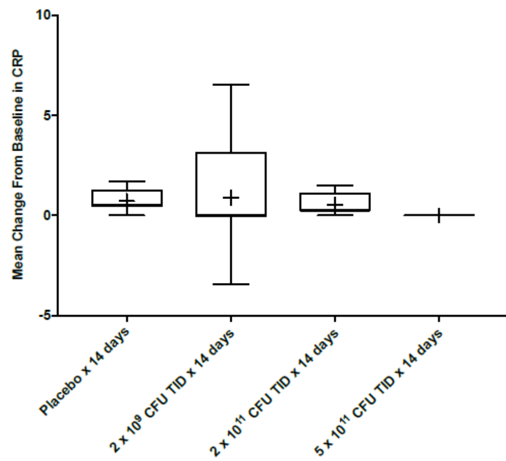

Fig. S6. Change from baseline in C-reactive protein. No change from baseline in mean CRP was observed in the multiple-dose cohorts. Placebo n=6, 2x10^9 CFU (n=6), 2x1010 CFU (n=6), 5x10^{10} CFU (n=5)

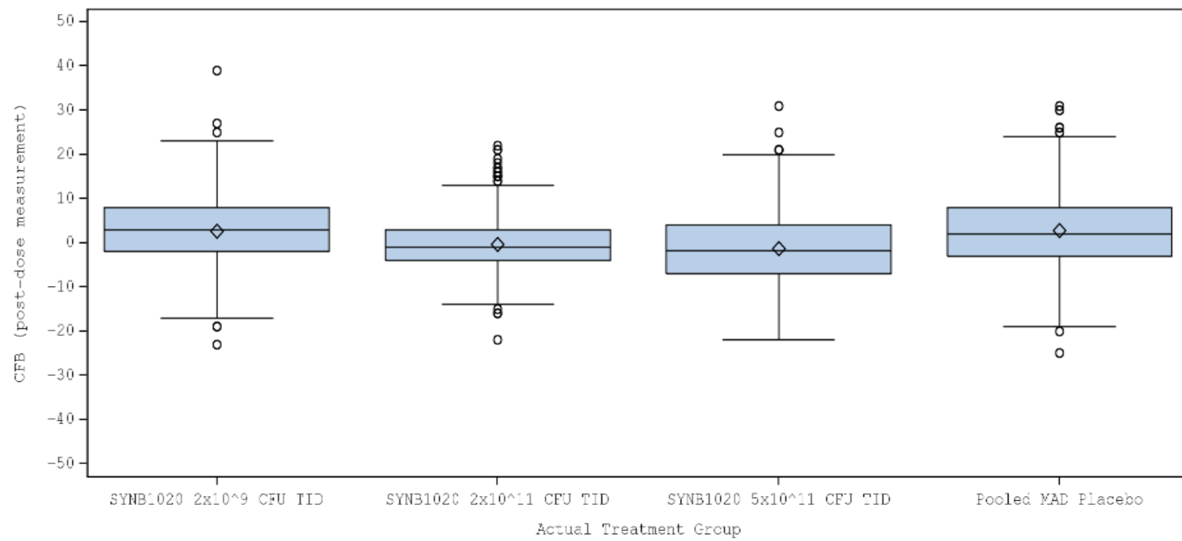

Fig. S7. Time-matched change from baseline for diastolic blood pressure. No change from baseline in mean diastolic BP was observed in the multiple-dose cohorts. Placebo n=6, $2x10^9$ CFU (n=6), $2x10^{10}$ CFU (n=6), $5x10^{10}$ CFU (n=5)

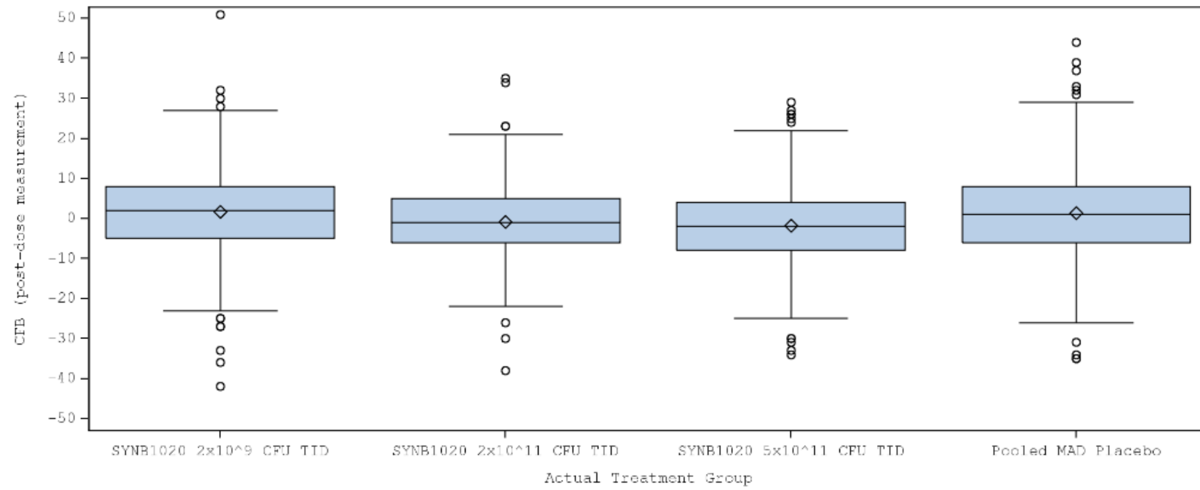

Fig. S8. Time-matched change from baseline for systolic blood pressure. No change from baseline in mean systolic BP was observed in the multiple-dose cohorts. Placebo n=6, $2x10^9$ CFU (n=6), $2x10^{10}$ CFU (n=6), $5x10^{10}$ CFU (n=5)

Table S1. Differential gene expression comparisons of SYNB1020 (A) and EcN$^{\Delta thyA}$ (B) to EcN. Provided as an Excel file.

Table S2. Quantification of viable SYNB1020^{KR} in mouse cecolonic samples over time.

Time Post Dose (hour)	SYNB1020^{KR} (1 x 10^{10} CFU)	SYNB1020^{KR} (1 x 10^{9} CFU)	SYNB1020^{KR} (1 x 10^{8} CFU)	SYNB1020^{KR} (1 x 10^{7} CFU)
0	2.10 x 10^{10}	2.70 x 10^{9}	1.60 x 10^{8}	1.90 x 10^{7}
1	2.70 x 10^{10}	2.10 x 10^{9}	1.20 x 10^{8}	1.70 x 10^{7}
2	1.80 x 10^{10}	1.60 x 10^{9}	7.20 x 10^{8}	1.00 x 10^{7}
3	1.00 x 10^{10}	2.20 x 10^{9}	1.90 x 10^{8}	1.30 x 10^{7}
4	1.30 x 10^{10}	1.60 x 10^{9}	1.20 x 10^{8}	2.40 x 10^{7}
20	5.00 x 10^{7}	1.50 x 10^{9}	2.30 x 10^{8}	2.60 x 10^{8}
44	2.00 x 10^{3}	1.50 x 10^{9}	2.30 x 10^{8}	6.00 x 10^{8}
68	0.00	8.90 x 10^{8}	1.50 x 10^{8}	4.90 x 10^{8}

Table S3. Average ECN^{StrR} or SYNB1020^{KR} CFU recovered from GI contents of mice dosed with each strain. Contents from cecum and colon were isolate from mice dosed with bacteria for 3 days and were plated on LB agar for quantification of bacteria present.(n=3/group).

		Cecum			Colon		
Strain	**Amount Dosed (CFU)**	**Average (CFU)**	**Standard Deviation**	**Percent Recovery**	**Average (CFU)**	**Standard Deviation**	**Percent Recovery**
EcN^{StrR}	3.7 x 10^{10}	2.57 x 10^{8}	3	0.69%	7.00 x 10^{7}	±2.75 x 10^{7}	0.19%
SYNB1020^{KR}	4.1 x 10^{10}	1.02 x 10^{9}	±1.19 x 10^{9}	2.50%	1.87 x 10^{8}	±1.63 x 10^{8}	0.46%

Table S4. Activity rate of SYNB1020^{KR} recovered from mouse cecum and colon. n=3/group.

	Cecum		**Colon**		**In Vitro**	
Strain	**Mean Rate** (nmoles arginine/10^9 cells/hour)	**Standard Error**	**Mean Rate** (nmoles arginine/10^9 cells/hour)	**Standard Error**	**Mean Rate** (nmoles arginine/10^9 cells/hour)	**Standard Error**
EcN^{StrR}	0.261	±0.243	0.498	±0.889	N/A	N/A
SYNB1020^{KR}	535.1	±105.1	374.3	±58.2	650	±17.0

Table S5. Arginine pathway metabolites in mouse and nonhuman primates. Female C57BL/6J mice (6-12 weeks old) or males OTC Spf-ash (6-12 weeks old) were orally administered bacteria with or without $^{15}N\text{-}NH_4Cl$ and maintained in metabolic cages for urine collection for the period indicated above. Blood was collected by submandibular bleeding at the indicated timepoints and processed for plasma extraction. Male cynomolgus monkeys (3-4kg) were orally administered SYNB1020 with or without $^{15}N\text{-}NH_4Cl$. Blood was collected throughout the study for up to 6hrs post administration and urine collected at the 6hr timepoint.

Animal	Strain	Dosing Regimen	Other treatment	Biomarker signal (mean ± SD), p value
C57BL/6	$SYNB1020^{KR}$	3 days BID + 1 day QD (1×10^{10} CFU /dose)	$^{15}N\text{-}NH_4Cl$ (0.25M) by enema	Urine $^{15}N\text{-}NO_3$ at 4hrs Vehicle: 0.00054 μmol ± 0.00015 (n=2), $SYNB1020^{KR}$: 0.00144 μmol ± 0.00071 (n=2), p=0.2217
C57BL/6	SYNB1020	3 days BID + 1 day QD (1×10^{10} CFU /dose)	$^{15}N\text{-}NH_4Cl$ (0.25M) by enema	Urine $^{15}N\text{-}NO_3$ at 4hrs Vehicle: 0.23 MPE ± 0.29 (n=3), SYNB1020: 0.23 MPE ± 0.11 (n=4), p=0.9749
C57BL/6	SYNB1020	3 days BID (1×10^{10} CFU /dose)		Plasma arginine 4hrs Vehicle: 0.021 mM ± 0.006 (n=9), SYNB1020: 0.026 mM ± 0.007 (n=9), p=0.1145. Plasma arginine 6hrs Vehicle: 0.017 mM ± 0.003 (n=9), SYNB1020: 0.025 mM ± 0.007 (n=9), p=0.0053. Plasma citrulline 4hrs Vehicle: 0.015 mM ± 0.002 (n=9), SYNB1020: 0.021 mM ± 0.004 (n=9), p=0.0015. Plasma citrulline 6hrs Vehicle: 0.015 mM ± 0.002 (n=9), SYNB1020: 0.020 mM ± 0.005 (n=9), p=0.0105. Plasma proline 4hrs Vehicle: 0.060 mM ± 0.009 (n=9), SYNB1020: 0.068 mM ± 0.026 (n=9), p=0.056463. Plasma proline 6hrs Vehicle: 0.050 mM ± 0.008 (n=9), SYNB1020: 0.071 mM ± 0.027 (n=9), p=0.04716. Urine orotate 4hrs Vehicle: 0.030 mM ± 0.010 (n=9), SYNB1020: 0.015 mM ± 0.011 (n=9), p=0.0084. Urine orotate 6hrs Vehicle: 0.020 mM ± 0.019 (n=9), SYNB1020: 0.010 mM ± 0.005 (n=9), p=0.01726.

$OTC^{spf-ash}$	SYNB1020	Single dose (1×10^{10} CFU /dose)		Urine orotate 2hrs Vehicle: 0.73 mM ± 0.30 (n=6), SYNB1020: 0.51 mM ± 0.22 (n=6), p=0.17465. Urine orotate 6hrs Vehicle: 0.42 mM ± 0.06 (n=6), SYNB1020: 0.46 mM ± 0.04 (n=6), p=0.274393.
C57BL/6	SYNB1020	Single dose (1×10^{10} CFU /dose)	Oral ^{15}N-NH_4Cl (0.4M)	Blood ^{15}N-NO_3 EcN: 0.032 MPE ± 0.052 (n=6), SYNB1020: 0.007 ± 0.074 (n=6), p=0.0509585. Urine ^{15}N-NO_3 EcN: 0.058 MPE ± 0.0100 (n=6), SYNB1020: 0.187 ± 0.026 (n=6), p=0.022198.
C57BL/6	SYNB1020	Single dose (1×10^{10} CFU /dose)		Blood arginine 4hrs EcN: 0.072 mM ± 0.043 (n=12), SYNB1020: 0.068 ± 0.032 (n=12), p=0.81239. Blood arginine 6hrs EcN: 0.058 mM ± 0.059 (n=12), SYNB1020: 0.053 ± 0.023 (n=12), p=0.02765. Blood citrulline 4hrs EcN: 0.033 mM ± 0.005 (n=12), SYNB1020: 0.032 mM ± 0.005 (n=12), p=0.795153. Blood citrulline 6hrs EcN: 0.028 mM ± 0.004 (n=12), SYNB1020: 0.028 mM ± 0.003 (n=12), p=0.998541. Blood orotate 4hrs EcN: 0.009 mM ± 0.006 (n=6), SYNB1020: 0.010 mM ± 0.011 (n=8), p=0.929609. Blood orotate 6hrs EcN: 0.010 mM ± 0.006 (n=8), SYNB1020: 0.018 mM ± 0.013 (n=10), p=0.09666.
$OTC^{spf-ash}$	SYNB1020	Single dose (1×10^{10} CFU /dose)		Urine orotate 2hrs Vehicle: 0.281 mM ± 0.368 (n=9), SYNB1020: 0.318 mM ± 0.136 (n=10), p=0.77. Urine orotate 4hrs Vehicle: 0.219 mM ± 0.160 (n=9), SYNB1020: 0.287 mM ± 0.183 (n=10), p=0.3984
NHP	SYNB1020	Single dose ($1\times$ CFU /dose)		Urinary NO_3 (corrected for creatinine) Vehicle: 3.71 μM/ mg/dL creatinine ± 1.264 (n=5), SYNB1020: 5.842 μM/ mg/dL creatinine ± 1.17 (n=5), p=0.0244
NHP	SYNB1020	Single dose (1×10^{12} CFU /dose)	Oral ^{15}N-NH_4Cl (60 mg)	Plasma ^{15}N-NO_3 AUC EcN: 0.08959 MPE · hr ± 0.1005 (n=5), SYNB1020: 0.8665 MPE · hr ± 0.5151 (n=5), p=0.177
NHP	EcN and SYNB1020	Single dose (1×10^{12} CFU /dose)	Oral ^{15}N-NH_4Cl (150 mg)	Plasma ^{15}N-NO_3 0.5hr EcN: 0.210 MPE ± 0.133 (n=5), SYNB1020: 0.186 MPE ± 0.118 (n=5), p=0.7707. Plasma ^{15}N-NO_3 1hr EcN: 0.286 MPE ± 0.113 (n=5), SYNB1020: 0.224 MPE ± 0.182 (n=5), p=0.5355. Plasma ^{15}N-NO_3 2hr EcN: 0.284 MPE ± 0.136 (n=5), SYNB1020: 0.139 MPE ± 0.079 (n=5), p=0.0711.

				Plasma $^{15}N\text{-}NO_3$ 4hr EcN: 0.17 MPE ± 0.152 (n=5), SYNB1020: 0.146 MPE ± 0.107 (n=5), p=0.7799. Plasma $^{15}N\text{-}NO_3$ 6hr EcN: 0.114 MPE ± 0.082 (n=5), SYNB1020: 0.172 MPE ± 0.070 (n=5), p=0.2629. Urine $^{15}N\text{-}NO_3$ 6hr EcN: 0.214 MPE ± 0.072 (n=5), SYNB1020: 0.298 MPE ± 0.050 (n=5), p=0.0647.
NHP	SYNB1020	3 days BID + 1 day QD (1×10^{11} CFU /dose)	Single oral $^{15}N\text{-}NH_4Cl$ (150 mg) on day 4	Plasma $^{15}N\text{-}NO_3$ Day 4 6hr Vehicle: 5.161 MPE ± 1.136 (n=9), SYNB1020: 3.989 MPE ± 1.900 (n=9), p=0.1361. Urine $^{15}N\text{-}NO_3$ Vehicle: 0.326 MPE ± 0.239 (n=9), SYNB1020: 0.262 MPE ± 0.414 (n=9), p=0.6925.

Table S6. Clinical and laboratory results from a 28-day oral toxicity study of SYNB1020 in mice. No clinically significant changes were observed in mice treated for 28 days with SYNB1020.

Species/Strain: CD-1	**Duration of Dosing**: 28 Days	**Sponsor Reference No.** SYN.001
Initial Age: 5-6 Weeks	**Duration of Postdose**: 14 Days	**Testing Facility Study No.** SNBL.1052.03
Date of First Dose: 30 Nov 2016	**Method of Administration**: Oral Gavage	**GLP Compliance**: GLP
	Vehicle/Formulation: 15% (v/v) glycerol, Sodium chloride, potassium chloride, sodium phosphate dibasic, potassium phosphate monobasic, sodium bicarbonate	
Special Features: Synthetic Biotic		
No Observed Adverse Effect Level: ~1.56×10^{11} CFU/day		

Dose (CFU/day)	0 (Control)		~5.2×10^{9} CFU/day		~5.2×10^{10} CFU/day		~1.56×10^{11} CFU/day	
Number of Animals/Sex/Group:	M	F	M	F	M	F	M	F
Main:	10	10	10	10	10	10	10	10
Recovery:	6	6	6	6	6	6	6	6
Noteworthy Findings								
Died or Euthanized Moribund	2	0	1	0	0	0	1	1
Clinical Observations	-	-	-	-	-	-	-	-
Body Weight								
Day14 (g)	32.8	26.1	-	-	-	-	-	-
Day 21 (g)	35.3	27.9	-	-	-	-	-	-
Day 28 (g)	35.3	27.4	-	-	-	-	-	-
Day 35 (g)	36.7	28.1	-	-	-	-	-	-
Food Consumption[c]								
Day 22 (g/animal/day)	7.9	6.5	-	-	-	-	-	-
Day 29 (g/animal/day)	7.4	5.6	-	-	-	-	-	-
Day 36 (g/animal/day)	6.2	6.0	-	-	-	-	-	-
Day 42 (g/animal/day)	5.3	5.8	-	-	-	-	-	-
Ophthalmology	-	-	-	-	-	-	-	-
Hematology	-	-	-	-	-	-	-	-
Coagulation	-	-	-	-	-	-	-	-
Serum Chemistry	-	-	-	-	-	-	-	-
Gross Pathology	-	-	-	-	-	-	-	-
Organ Weights	-	-	-	-	-	-	-	-
Histopathology	-	-	-	-	-	-	-	-

Abbreviations:
- = no noteworthy findings

Table S7. Mice with detectable SYNB1020 DNA in tissues as measured by qPCR. Tissues were collected from male and female mice during the terminal necropsy, on Day 29. Each dose group initially comprised 16 males and 16 females; however, 6 unscheduled necropsies occurred (3 in the control, 1 in the 5×10^{9} CFU group, and 2 in the 1.5×10^{11} CFU group) and were considered not related to treatment.

Group	Gender	Stomach	Duodenum	Jejunum	Ileum	Colon	Spleen	Liver	Bladder	Ovary	Testes
Vehicle	M	0/13	0/13	0/13	0/13	0/13	0/13	0/13	0/13	NA	0/13
	F	0/16	0/16	0/16	0/16	0/16	0/16	0/16	0/16	0/16	NA
5×10^{9} CFU	M	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	NA	0/15
	F	[a]1/16	0/16	0/16	0/16	0/16	0/16	0/16	0/16	0/16	NA
1.5×10^{11} CFU	M	0/15	0/15	0/15	0/15	[b]1/15	0/15	0/15	0/15	NA	0/15
	F	0/15	0/15	0/15	[c]1/15	0/15	0/15	0/15	0/15	0/15	NA

Abbreviations: CFU = colony-forming units; F = female; M = male; NA = not applicable

[a] 1/16 stomachs had detectable SYNB1020 DNA (48.6 ± 2.3 copies).

[b] 1/15 ileums had detectable SYN1020 DNA (10.3 ± 1.6 copies).

[c] 1/15 colons had detectable SYNB1020 DNA (41 ± 6.4 copies).

Table S8. Baseline demographics for the phase 1 study population.

	Single Day Cohorts							Multiple Day Cohorts			Pooled Placebo Cohorts		
	2×10^{9} CFU QD (N=3)	2×10^{10} CFU QD (N=3)	2×10^{11} CFU QD (N=3)	2×10^{12} CFU QD (N=3)	5×10^{11} CFU TID (N=3)	1×10^{12} CFU TID (N=3)	2×10^{12} CFU TID (N=3)	2×10^{9} CFU TID (N=6)	2×10^{11} CFU TID (N=6)	5×10^{11} CFU TID (N=6)	SAD Part (N=7)	MAD Part (N=6)	Total (N=52)
Age (years)													
Mean ± SD	30.3±9.5	51.7±10.5	48.7±18.9	58.3±5.5	28.7±5.9	37.0±14.7	28.0±4.6	40.5±11.0	44.3±9.1	36.7±11.6	39.7±13.5	39.5±10.0	40.2±12.5
Median (range)	30.0 (21-40)	52.0 (41-62)	57.0 (27-62)	58.0 (53-64)	31.0 (22-33)	34.0 (24-53)	27.0 (24-33)	40.5 (24-53)	43.5 (34-56)	30.5 (28-53)	30.0 (28-60)	41.5 (23-49)	37.5 (21-64)
Gender, n (%)													
Female	1 (33)	1 (33)	1 (33)	2 (67)	1 (33)	1 (33)	0	1 (17)	1 (17)	0	0	3 (50)	12 (23)
Male	2 (67)	2 (67)	2 (67)	1 (33)	2 (67)	2 (67)	3 (100)	5 (83)	5 (83)	6 (100)	7 (100)	3 (50)	40 (77)
Ethnicity, n (%)													
Hispanic or Latino	0	0	1 (33)	0	0	0	0	0	0	1 (17)	0	1 (17)	3 (6)
Not Hispanic or Latino	3 (100)	3 (100)	2 (67)	3 (100)	3 (100)	3 (100)	3 (100)	6 (100)	6 (100)	5 (83)	7 (100)	5 (83)	49 (94)
Race, n (%)													
American Indian or Alaska Native	0	0	0	1 (33)	0	0	0	0	0	0	0	0	1 (2)
Black or African American	3 (100)	2 (67)	1 (33)	1 (33)	3 (100)	2 (67)	2 (67)	5 (83)	3 (50)	5 (83)	6 (86)	4 (67)	37 (71)
White	0	1 (33)	2 (67)	1 (33)	0	1 (33)	1 (33)	1 (17)	3 (50)	1 (17)	1 (14)	2 (33)	14 (27)
BMI (kg/m^2)													
Mean ± SD	24.5±3.2	25.6±2.4	27.0±1.4	28.5±1.3	24.2±1.5	26.4±1.6	21.0±2.3	25.2±2.8	27.6±1.1	24.5±3.2	26.7±1.6	24.7±3.4	25.6±2.7
Median (range)	24.4 (21.3-27.7)	26.3 (23.0-27.6)	27.6 (25.4-28.1)	29.0 (27.0-29.5)	25.0 (22.5-25.1)	27.1 (24.6-27.6)	20.2 (19.3-23.6)	25.8 (20.2-27.6)	27.8 (26.1-28.9)	24.8 (20.8-28.1)	26.5 (24.2-29.3)	26.0 (19.2-28.5)	26.3 (19.2-29.5)

Table S9. ECG QT interval in the phase 1, multiple-day, study population. ECG QT interval with Fridericia's correction (QTcF) change from baseline (Day 1 predose to Day 2). Values are mean [standard deviation].

ECG Assessment (units)	**Pooled Placebo (N=6)**	**2×10^9 CFU TID (N=6)**	**2×10^{11} CFU TID (N=6)**	**5×10^{11} CFU TID (N=5)**
QTcF (msec)	-0.89 [5.31]	-0.33 [6.75]	1 [7.22]	-4.44 [5.01]

Table S10. Laboratory results for urea cycle products in the multiple-day cohort phase 1 study population. Plasma urea, nitrogen and citrulline and urinary urea and orotic acid were measured at predose and during the dosing period in the multiple day dosing cohort. Values are mean [standard deviation].

Plasma Analyte (matrix, units)	**Timepoint (change over study days)**	**Pooled Placebo (N=6)**	**2×10^{9} CFU TID (N=6)**	**2×10^{11} CFU TID (N=6)**	**5×10^{11} CFU TID (N=5)**
Urea (urine, g/day)	Day -1 to Day 13	0.5 [1.22]	-1.5 [3.56]	0.33 [1.21]	0.6 [3.21]
Urea Nitrogen (blood, mg/dL)	Day 1 (predose) to Day 14	-1.17 [2.4]	1.67 [1.03]	2 [1.26]	0 [1.58]
Orotic Acid (urine, mmol/mol Cr)	Day -2 to Day 14	0.07 [0.29]	0.17 [0.08]	0.08 [0.13]	0.02 [0.11]
Arginine (blood, umol/L)	Day -2 to Day 14	-2.67 [15.45]	-3.5 [11.36]	-11.33 [20.97]	1 [23.21]
Citrulline (blood, umol/L)	Day -2 to Day 14	-6 [7.87]	0 [3.85]	-3.67 [4.41]	-4.8 [7.5]

Table S11. ^{15}N metabolites in the phase 1 study population. Time-matched change from baseline (Day -1 to Day 13) in ^{15}N metabolites in multiple day dosing cohort Phase 1 study population. Values are mean [standard deviation].

^{15}N Analyte (matrix, units)	**Timepoint (h)**	**Pooled Placebo (N=6)**	**2×10^9 CFU TID (N=6)**	**2×10^{11} CFU TID (N=6)**	**5×10^{11} CFU TID (N=5)**
^{15}N Urea (plasma, mole percent excess)	**0**	0 [0]	0 [0]	0 [0]	0 [0]
	4	-0.4617 [1.035]	-0.5883 [0.7568]	-0.5633 [0.7258]	0.744 [0.6063]
	11	-0.3433 [1.305]	-0.6233 [0.7873]	-0.3467 [0.5054]	0.306 [0.5173]
	24	0.09167 [0.5778]	-0.7233 [0.344]	-0.1300 [0.4854]	0.188 [0.222]
^{15}N Citrulline (plasma, mole percent excess)	**0**	0 [0]	0 [0]	0 [0]	0 [0]
	4	-1.758 [1.255]	-1.268 [1.137]	-0.8100 [0.683]	-0.5500 [0.8448]
	11	-0.7483 [1.553]	-0.6467 [0.8717]	-0.3650 [0.7787]	-0.06200 [1.138]
	24	0.04667 [1.505]	-0.2317 [0.6931]	-0.3667 [0.6065]	1.508 [0.6983]
^{15}N Urea (urine, mole percent excess)	**0**	0 [0]	0 [0]	0 [0]	0 [0]
	4	-0.1767 [3.425]	0.865 [1.105]	-1.475 [4.275]	0.668 [1.576]
	11	-1.593 [1.526]	-0.2500 [1.233]	-0.7817 [1.158]	-0.0320 [0.5193]
	24	-0.4933 [1.168]	-0.6383 [1.006]	-0.3733 [0.525]	0.188 [0.4279]

Table S12. Primers used to characterize SYNB1020 genetic modifications. Primers used to delete the *thyA* gene and to insert the feedback resistant *argA* (*argA*^{fbr}) gene

Primer Name:	Primer Sequence:	Purpose:
*thyA::cm*_Forward	5'-TCGTCGCAGCCCACAGCAACACGTTTCCTGAGGAACCAGCCATGGTCCATATGAATATCC	Knock out of *thyA* gene
*thyA::cm*_Reverse	5'-CGACGCACACTGGCGTCGGCTCTGGCAGGATGTTTCGTAATCTTGAGCGATTGTGTAGGC	
*malE_argA*_Forward	5'-CCATAATTCACTTTACTGGTG	Knock in of *argAfbr* gene
*malK_argA*_Reverse	5'-TCAGCGATATCACTCGGC	

Table S13. Fermentation medium (FM1). Media used to prepare bacterial strains.

Component:	Amount Required per L:
Yeast extract	25 g
Potassium phosphate, dibasic (K_2HPO_4)	5 g
Potassium phosphate, monobasic (KH_2PO_4)	3.5 g
Ammonium phosphate, dibasic ($(NH_4)_2HPO_4$)	3.5 g
Magnesium sulfate, heptahydrate ($MgSO_4$ $7xH_2O$)	0.5 g
Glucose	25 g
Antifoam 204	125 µL
Thymidine	10 mM
Ferric Chloride ($FeCl_3$)	1.6 x 10^{-3} g
Cobaltous chloride, hexahydrate ($CoCl_2$ $6H_2O$)	0.2 x 10^{-3} g
Cupric chloride (CuCl2)	0.1 x 10^{-3} g
Zinc Chloride ($ZnCl_2$)	0.2 x 10^{-3} g
Molybdic acid sodium salt, dihydrate ($NaMoO_4$)	0.2 x 10^{-3} g
Boric Acid (H_3BO_4)	0.05 x 10^{-3} g
Water	*Quantum satis* to 1 L

Abbreviations: g= grams; L = liter; µL = microliters; mM = millimolar