Supplementary Material for:

Genetic manipulation of gut microbes enables single-gene interrogation in a complex microbiome

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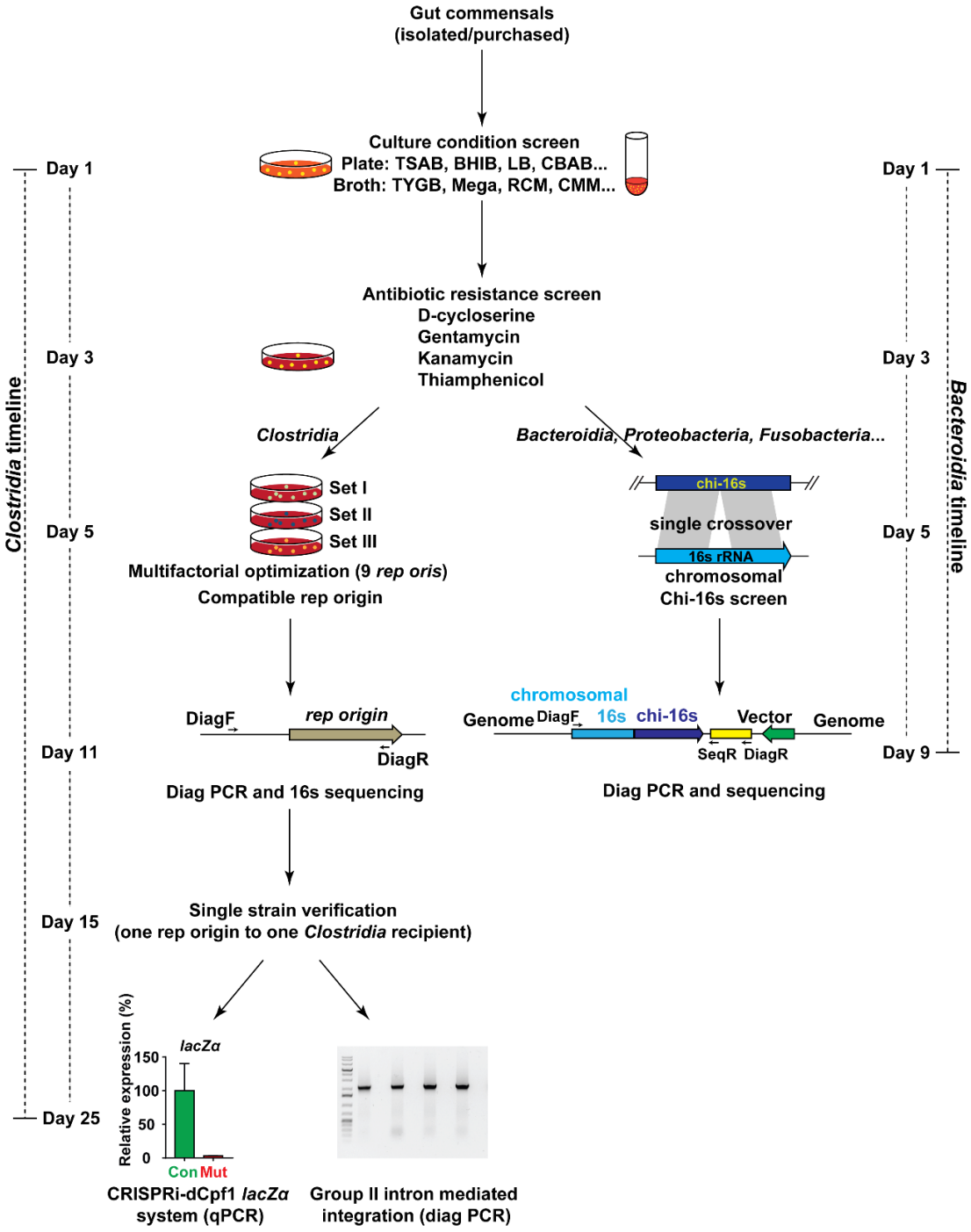
Table of Contents

SUPPLEMENTARY DATA FILE S1

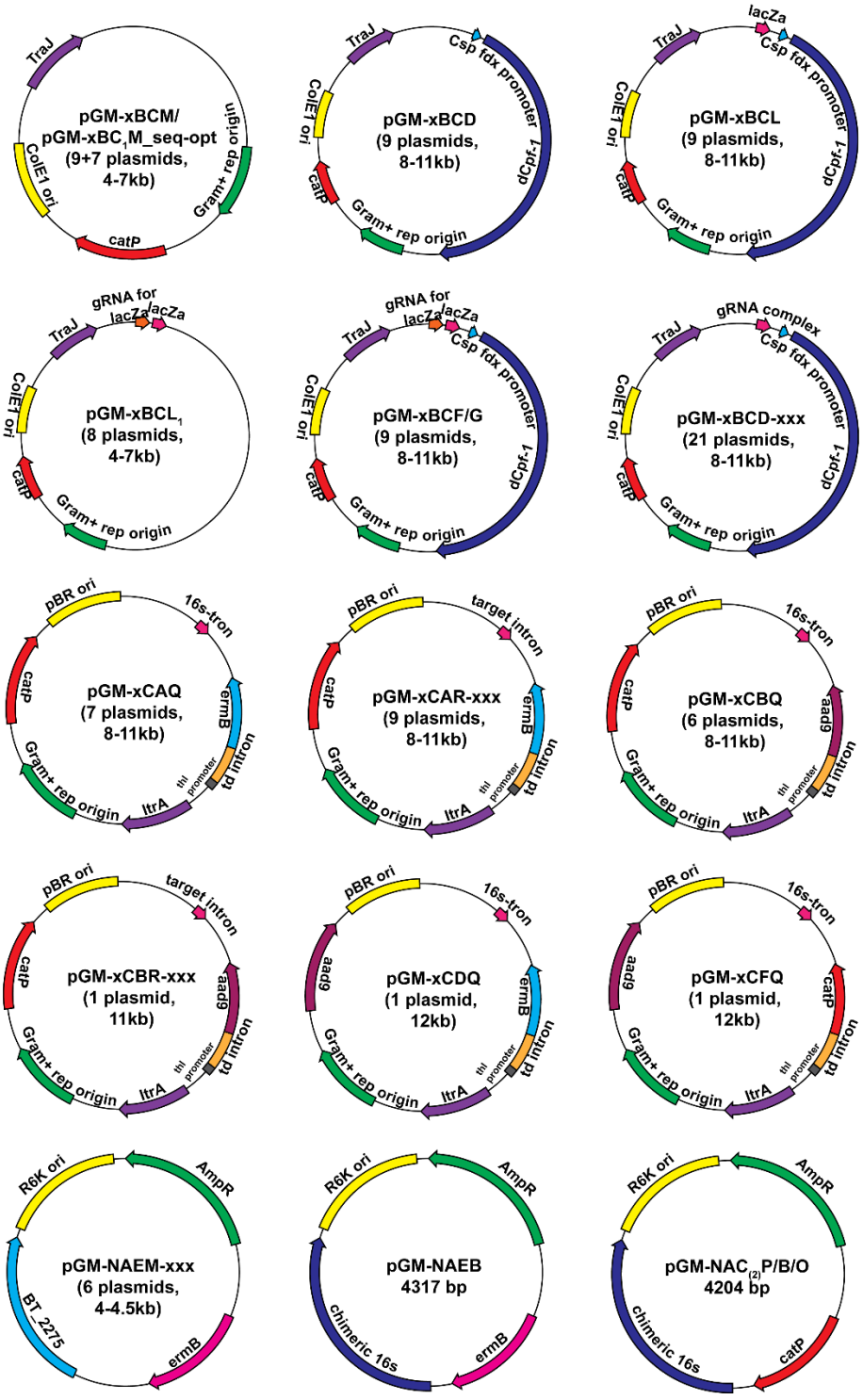
Section I. An estimated timeline of the GM pipeline

An estimated timeline and the plasmids used in this study are shown in the figures below.



Data S1A. A detailed workflow and general timeline of the genetic manipulation (GM) pipeline, related to Figure 1

The non-model human gut commensals can be screened via the GM pipeline, and their targetable genetic system can be built within weeks.



Data S1B. All the pGM vectors used in this study, related to Figure 2

Schematics of all the pGM vectors designed and used in this study are listed. “x” in pGM-xBCM (or pGM-xBCD, pGM-xBCL, pGM-xBCF/G, pGM-xBCD-xxx) represents different gram-positive replication origins,

and “xxx” in pGM-xBCD-xxx corresponds to plasmid pGM-xBCD harboring different gRNA designs targeting genome of different strains (see **Table S2** for detailed information and nomenclature).

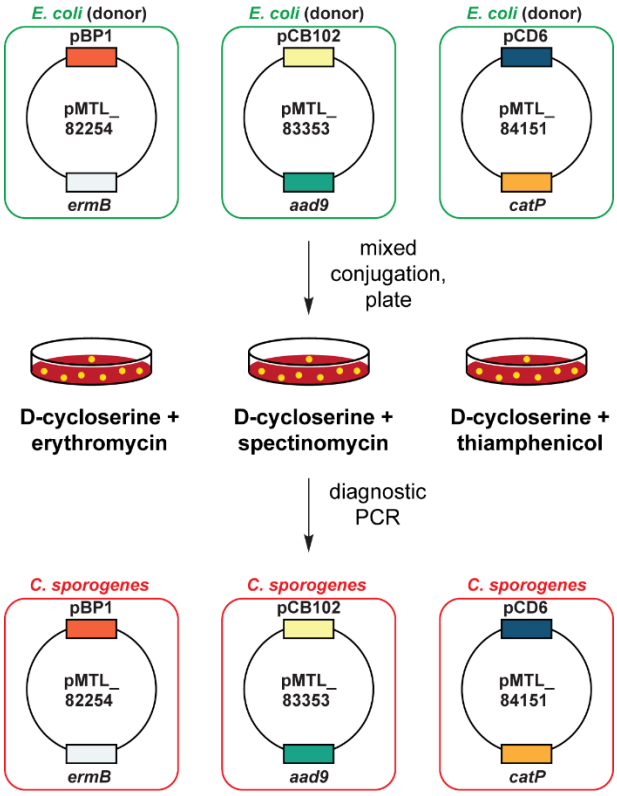
Section II. Sequence optimization

The set of *Clostridia* conjugation plasmids was sequence-optimized by **1)** codon-optimizing the coding sequences (CDSs) of *catP*, *traJ*, and *Clostridial rep oris* to reduce their putative *Clostridial* Type II-RM sites (**Table S2**), and **2)** replacing the *catP* promoter $P_{pmtl\text{-}catP}$ with P_{fdx} (the most potent promoter identified via a promoter screen). In brief, we searched the REBASE database and found 22 cutting sites that are most often recognized by the Type II-RM of *Clostridia* bacteria (including the solventogenic *Clostridium* genus), putative RM sites reduced in the sequence optimization include: AAGCTT, ACACAG, ACTGGG, AGGCCT, CAGCTG, CCCGGG, CCCGT, CCGG, CCNGG, CCSSGG, CCWGG, CTAG, CTGCAG, GAATTC, GACGC, GASTC, GATC, GCGC, GCNGC, GGCC, GGNCC, GTCGAC. Then the CDSs of *catP*, *traJ*, and *rep oris* were codon-optimized to reduce the number of these restriction sites by at least half. Of note, the promoter and terminator of the CDS like *catP* or *traJ* and some highly repetitive motifs in the *rep oris* were left untouched. These sequences play a key role in regulating the functions of *catP* and *rep ori*, and any mutation (or nucleotide switch) could potentially cause dysfunction of *catP* or *rep ori* and lead to unsuccessful transformation. These plasmids are labeled with ‘seq-opt’. Please refer to **Table S1** for the *Clostridia* that uptakes this set of vectors. All the gene transfer conditions via conjugations/transformations have been repeated at least three times in our study.

Section III. Test the mixed-conjugation strategy in *C. sporogenes* ATCC 15579 (S107)

A preliminary test was performed to assess if a model gut commensal **S107** can uptake plasmids with a compatible *rep ori* from three *E. coli* conjugation donors in one conjugation (**Data S1C**). We inoculated three *E. coli* HB101/pRK24 donors harboring three different vectors pMTL82254 (*rep ori*: pBP1; antibiotic: erythromycin), pMTL83353 (*rep ori*: pCB102; antibiotic: spectinomycin), and pMTL84151 (*rep ori*: pCD6; antibiotic: thiamphenicol), respectively. **S107** was inoculated in 1 mL TYGC liquid broth and grown anaerobically at 37 °C for 12~18 hrs. The three *E. coli* donors were inoculated into LB liquid broth supplemented with the corresponding antibiotics (erythromycin: 250 μg/mL; spectinomycin: 100 μg/mL; chloramphenicol: 25 μg/mL) and shaken at 220 rpm for overnight. The next day, 700 μL of each *E. coli* culture were mixed and centrifuged at 1500 x *g* for 2 min. The cell pellet was washed with 1.5 mL PBS (pH 7.4) and centrifuged again at 1500 x *g* for 2 min. The PBS supernatant was removed, and the cell pellet was transferred on ice into the anaerobic chamber. The cell pellet was mixed with 300 μL of the overnight **S107** culture, and a 35 μL cell mixture was dotted on pre-reduced TYG agar plates. After 48 hrs, the cell dots were scraped using a sterile inoculation loop and resuspended in 300 μL pre-reduced PBS (pH 7.4) buffer. 50 μL of the cell suspension was plated onto three TYG agar plates that were supplemented with

D-cycloserine (250 μg/mL) + erythromycin (10 μg/mL, to select for pMTL82254), or spectinomycin (500 μg/mL, to select for pMTL83353), or thiamphenicol (15 μg/mL, to select for pMTL84151).



Data S1C. Test of the mixed-conjugation strategy in S107, related to Figure 2

A preliminary test of the mixed-conjugation strategy was performed in a model gut commensal **S107**. The *E. coli* conjugation donors each harboring a single *Clostridium rep ori* and antibiotic marker gene (pMTL82254, *rep ori*: pBP1, antibiotic marker: *ermB*, erythromycin; pMTL83353, *rep ori*: pCB102, antibiotic marker: *aad9*, spectinomycin; pMTL84151, *rep ori*: pCD6, antibiotic marker: *catP*, thiamphenicol) were mixed and conjugated to a single recipient **S107**. After conjugation, the transconjugants were selected on agar plates supplemented with D-cycloserine and the one corresponding antibiotic (erythromycin for *ermB*, spectinomycin for *aad9*, and thiamphenicol for *catP*).

Section III. Modulating *Clostridia bcat* expression and microbiome-derived metabolites using gene manipulation tools developed via the GM pipeline.

1. Targeted suppression of BCAA aminotransferase (*bcat*) gene and modulate butyrate production in non-model gut *Clostridia*

Using the strain **S72** as an example. The assembled vectors pGM-FBCD and pGM-FBCD-010 were transformed into chemically competent *E. coli* CA434, respectively. *E. coli* CA434 harboring pGM-FBCD and pGM-FBCD-010 were conjugated to S72. The transconjugants were picked and restreaked onto TSAB supplemented with D-cycloserine (250 μg/mL) + thiamphenicol (15 μg/mL). Then, three isolated

single colonies were cultivated in 5 mL Mega broth supplemented with D-cycloserine (250 μg/mL) + thiamphenicol (15 μg/mL) for 36 hrs, the RNA was extracted, and qPCR was performed to quantify the relative expression of *bcat* after normalizing to 16S rRNA gene (**Table S2, Fig. S3**).

To deplete *croA* in *Clostridia* strains utilizing Group II intron, the assembled plasmid pGM-FCAR-003 targeting *croA* in **S115** was introduced into **S115** via conjugation (**Table S1**). Next, four transconjugants were restreaked onto a TSAB plate with 9 μg/mL thiamphenicol and 200 μg/mL gentamycin to isolate a single colony. The single colonies were inoculated into 1 mL Mega supplied with the same antibiotics. After 24-36 hrs, 1 mL of cultures were spread onto TSAB plates supplemented with 200 μg/mL gentamycin and 10 μg/mL erythromycin. The integrated colonies typically appeared after 48-72 hrs. Eight colonies were picked to inoculate 3 mL Mega supplemented with 200 μg/mL gentamycin and 10 μg/mL erythromycin. After 24-36 hrs, the genomic DNA was extracted diagnostic PCR was performed.

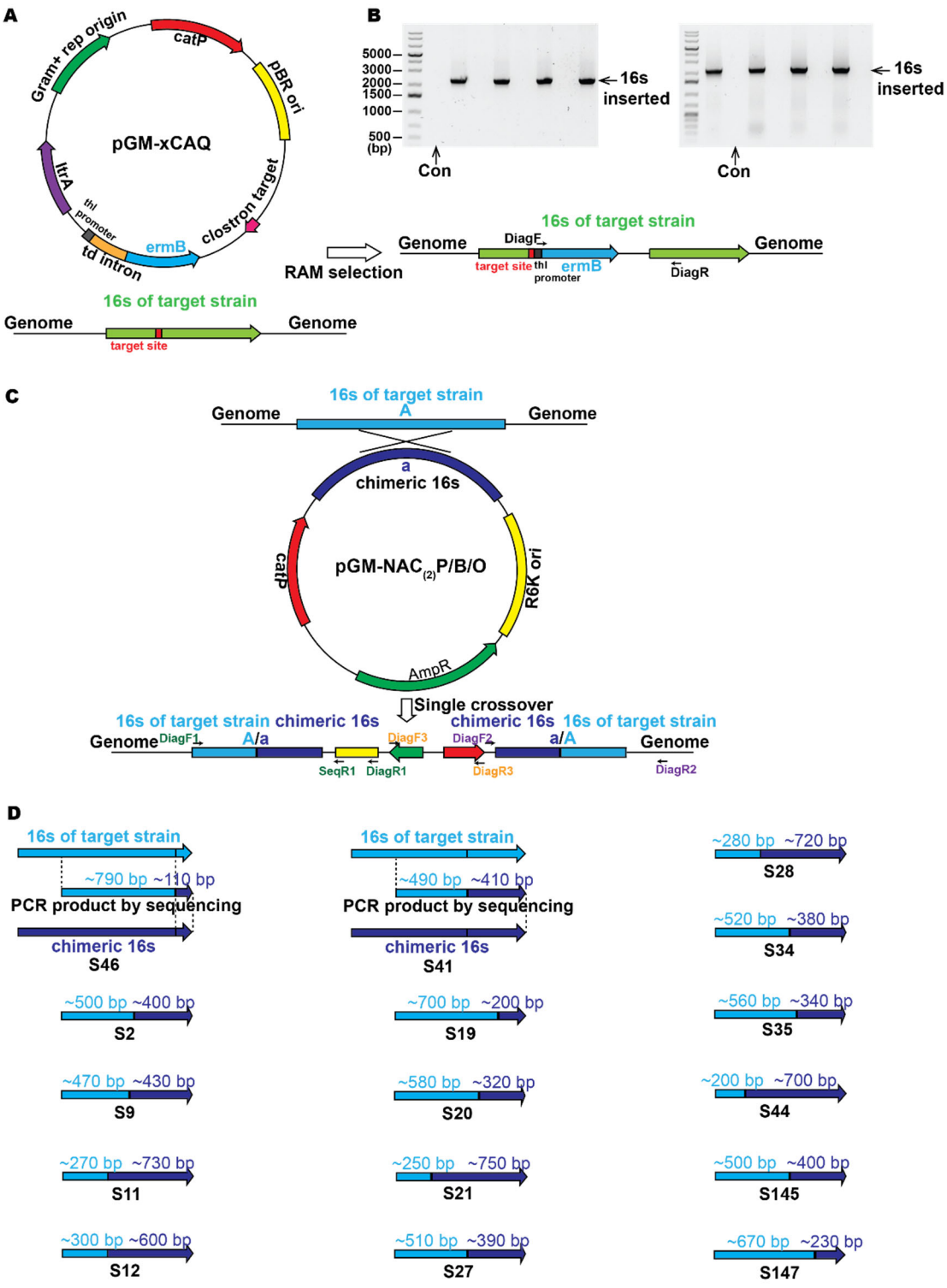
The butyrate production was evaluated by glucose assay with PBS washed cell of control, and *croA* mutant, 3 mL of culture was first centrifuged at 1500 x *g* for 3 min. The cell pellet was washed twice with 1 mL PBS (pH 7.4) and centrifuged again at 1500 x *g* for 3 min. The PBS supernatant was removed, and the cell pellet was resuspended with 500 μL PBS, and then glucose was added to the concentration of 1 mM. The mixture was incubated anaerobically at 37 °C for 1 h. The PBS suspension was subjected to SCFAs derivatization and LCMS measurement.

2. Genetic manipulation of *Bacteroidia* strains to deplete propionate production

Taking **S25** as an example, the assembled plasmid pGM-NACM-003 was introduced into **S25** via *E. coli* conjugation. About 48 hrs after plating the conjugation cell mixture, four colonies were picked and restreaked on a TSAB plate + 200 μg/mL gentamycin + 15 μg/mL thiamphenicol to isolate single colonies. A single colony was inoculated in 3 mL TYBG broth with the same antibiotics, and the bacterial genomic DNA was extracted. The diagnostic PCR was performed to verify the single crossover integration of pGM-NACM-003 into *mmdA* (**Data S1D**).

3. Targeted suppression of *porA* gene in **S107**

The assembled vectors pGM-ABCD (control) and pGM-ABCD-006 (*porA* transcription repression mutant) were introduced into **S107** via conjugation. For each conjugation, three isolated single colonies were cultivated in 5 mL TYGC liquid broth supplemented with D-cycloserine (250 μg/mL) + thiamphenicol (15 μg/mL) for 36 hrs. Then RNA was extracted, and the relative expression of *porA* in the control strain and its transcription repression mutant was quantified. To quantify the production of branched short-chain fatty acid, 10 μL supernatant of both the control strain and the *porA* transcription repression mutant was derivatized and subject to LC-qTOF analysis.



Data S1D. Diagnostic PCR strategy, related to Figures 2 and 3

(A) Diagnostic PCR strategy to verify the 16S-targeting Group II intron (16S-tron) retrotransposition-activated marker (RAM) integration designed in targeted *Clostridia* commensals. The forward diagnostic primer is the sequence on the retrotransposition-activated marker, which will not bind to the genome. The

reverse diagnostic primer binds to the genome and will not bind to the Group II intron plasmid. There will be a PCR product of 2.0-2.5 kb as designed for colonies that have integrated the retrotransposition-activated marker, whereas no PCR product will be found for control colonies.

(B) Two representative gel images of 16S-targeting Group II intron integration in *Clostridia*. There were bands of ~2.0-2.5 kb in colonies after RAM integration using primers described in (**A**), while no band was found in colonies of control.

(C) Diagnostic PCR strategy to verify the single crossover designed in targeted *Bacteroidia* commensals and microbes from other phyla. The genomic DNA of the wild-type microbe and the integration plasmids were used as controls. The diagnostic PCR strategy is the same for identifying the genetically targetable *Bacteroidia* and other phyla microbes using chi-16S strategy and for targeted deletion of *mmdA* in three *Bacteroidia* microbes to deplete propionate production. One diagnostic PCR primer is the sequence on the target gene of the genome (16S rRNA gene or *mmdA*), which will not bind to the introduced suicide plasmid, the other diagnostic PCR primer binds to the suicide plasmid-specific sequence and will not bind to the genome of targeted strains. There will be a PCR product of ~2.0-2.5 kb (from diagnostic primer pairs DiagF1 + DiagR1 and DiagF2 + DiagR2) as designed for colonies that have integrated the suicide plasmid, whereas no PCR product will be found for wild-type colonies. Also, diagnostic PCR was conducted with primers DiagF3 + DiagR3 to validate the existence of antibiotic marker integrated, and DiagF2 + DiagR3 to confirm the integration of suicide plasmid and no intact plasmid in the recipient microbe. For screening using chi-16S, the sequencing primers DiagF1 and SeqR1 were used for the PCR fragment from DiagF1 + DiagR1, sequencing primers DiagF2 and DiagR2 were used for the PCR fragment from DiagF2 + DiagR2. As the chimeric 16S is integrated into the genome, the nucleotide sequence (from Sanger sequencing) consists of part of the original 16S rRNA sequence and part of the chimeric 16S rRNA sequence.

(D) Alignment of the nucleotide sequence of the PCR product amplified using DiagF and DiagR (as shown in **(C)**) with the chimeric 16S rRNA sequence and the microbial 16S rRNA sequence for the genetically targetable *Bacteroidia* and microbes from other phyla identified using chi-16S. The number shown is a rough estimation of the integration position based on Sanger sequencing and alignment.

The numbering of the strains corresponds to the strain information shown in **Table S1**. All the PCR fragments were purified and sent for Sanger sequencing.

Section IV. Genetic disruption of *baiH* in *Faecalicatena contorta* S122 (S122)

To disrupt the *baiH* gene, *baiH*-targeting plasmid pGM-FCAR-002 was first introduced into **S122** via conjugation. Next, four transconjugants were restreaked onto a TSAB plate with 15 μg/mL thiamphenicol and 250 μg/mL D-cycloserine to isolate a single colony. The single colonies were inoculated

into 1 mL Mega supplied with the same antibiotics. After 24-36 hrs, 50 µL of cultures were spread onto TSAB plates supplemented with 250 µg/mL D-cycloserine and 10 µg/mL erythromycin. The integrated colonies typically appeared after 36-48 hrs. Eight colonies were picked to inoculate 3 mL Mega supplemented with 250 µg/mL D-cycloserine and 10 µg/mL erythromycin. After 24-36 hrs, the genomic DNA was extracted, and diagnostic PCR was performed (**Fig. S4C**).

To confer the *baiH* mutant strain with thiamphenicol resistance, the plasmid pGM-FCFQ was first introduced into three independent isolates of **S122** Ω*baiH* via conjugation. After conjugation, the **S122**+pGM-FCAR-002 colonies harboring pGM-FCFQ appeared on the TSAB plate supplemented with 10 µg/mL erythromycin, 300 µg/mL spectinomycin, and 250 µg/mL D-cycloserine. Next, four colonies were restreaked onto a TSAB plate with the same antibiotics to isolate single colony. The single colonies were inoculated into 1 mL Mega supplied with the same antibiotics. After 24-36 hrs, 50 µL of cultures were spread onto TSAB plates supplemented with 250 µg/mL D-cycloserine, 15 µg/mL thiamphenicol, and 10 µg/mL erythromycin. The integrated colonies typically appeared after 36-48 hrs. Colonies were picked to inoculate Mega supplemented with 250 µg/mL D-cycloserine, 15 µg/mL thiamphenicol, and 10 µg/mL erythromycin.

Likewise, the **S122** control strain with both erythromycin and thiamphenicol resistance was constructed using 16S-targeting plasmid pGM-FCAQ and pGM-FCFQ. The engineered strains with thiamphenicol and erythromycin resistance were validated and still carry at least one copy of intact 16S rRNA gene in their genomes by diagnostic PCR.

Section VI. Isolation of gut bacterial strains from collected fecal samples

Fecal samples (from human or mouse) were suspended in PBS (1:10 w/v), the suspension was then restreaked on TSAB/BHIB plates and incubated in an anaerobic chamber at 37 °C. In the meantime, the suspension was also restreaked on LB plates and incubated aerobically at 37 °C. Colonies typically appeared after 24-36 hrs. The isolated single colonies were inoculated in 3 mL Mega/RCM/CMM/TYBG or LB broth. After ~12 hrs, we extracted their genomic DNA and amplified the 16S rRNA region of the colony using primers 16S_27F + 16S_1391R. The PCR product was purified and sent for Sanger sequencing to identify the species of the isolated strains.

Section VII. Colonize germ-free and SPF mice with the control and mutant bacteria

Three isolates of control and mutant strains were prepared and mixed before colonization. For mono-colonization in germ-free mice, taking *Eubacterium maltosivorans* DSM 105863 control (S117+pGM-FBCD) and its SCFAs mutant (S117+pGM-FBCD-020) as an example, a 200 µL portion of their overnight RCM culture (~1 x 10^7 CFU) were mono-colonized with germ-free mice (n = 4 per group) via oral gavage. The germ-free mice were maintained on standard chow and water containing minimal thiamphenicol (15 µg/mL). Successful colonization was determined by colony-forming unit (CFU) counting. After two weeks

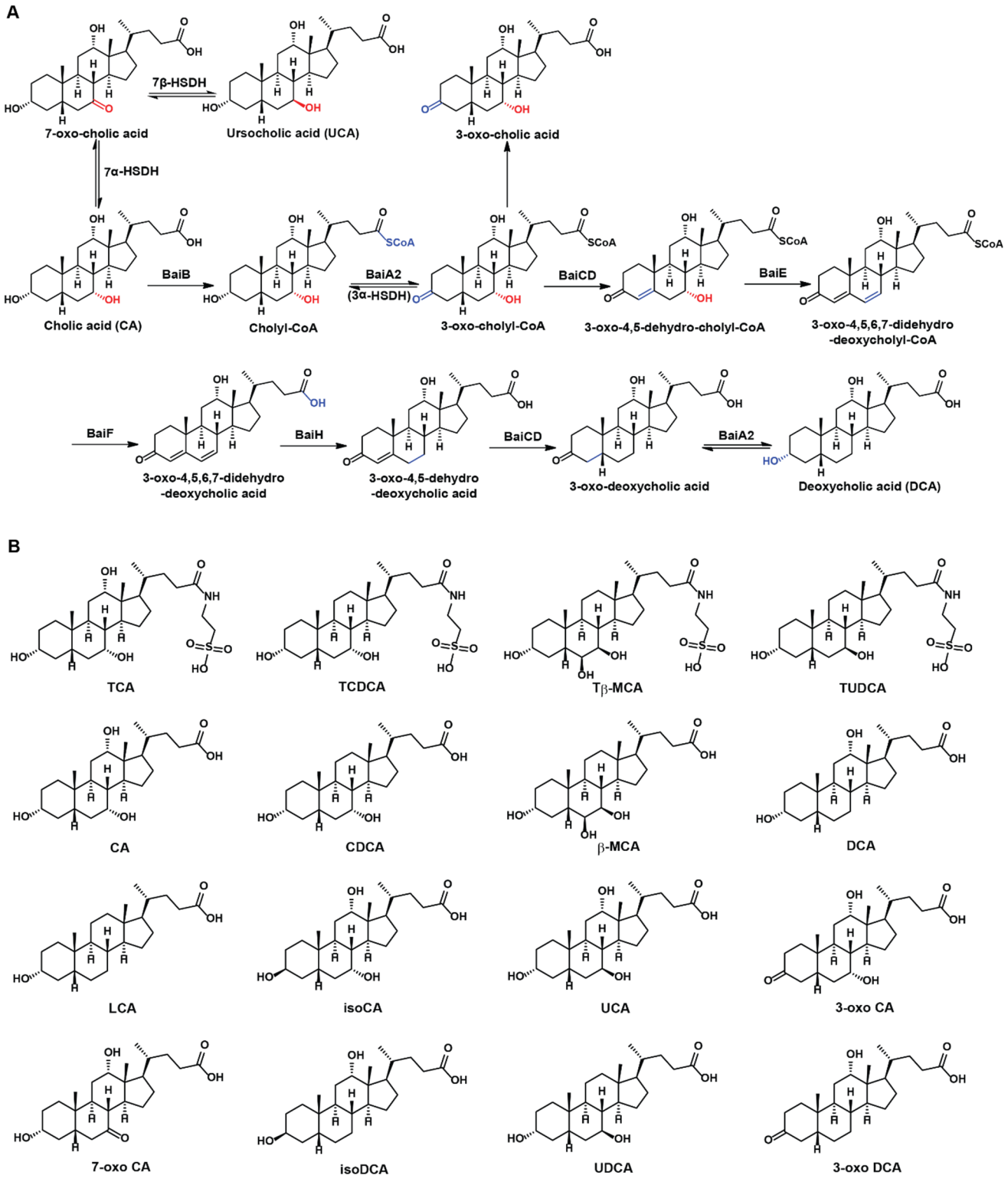
of colonization, mice were euthanized humanely by CO_2 asphyxiation. Blood was collected by cardiac puncture, and serum was prepared using microtainer serum separator tubes obtained from Becton Dickinson (Cat. # 365967). The urine, cecal contents, and feces were collected and snap-frozen in liquid nitrogen and stored at −80 °C until use.

For co-colonization of **S122** control or its Ω*baiH* mutant together with **S25** in germ-free mice, 1 mL of their overnight Mega culture (~1 x 10^7 CFU) were mixed and co-colonized with germ-free mice (n = 4 per group) via oral gavage (300 µL per mouse). The germ-free mice were maintained on standard chow and water supplemented with 15 µg/ml thiamphenicol. Successful colonization was determined by CFU plating, diagnostic PCR using their strain-specific primers, and 16S rRNA sequencing.

For co-colonization of **S122** control or its *baiH* mutant together with two *Bacteroides* (3-member community) mentioned in **Fig. S7G**, and co-colonization of **S122** control or its *baiH* mutant together with two *Bacteroides* (*Bac1-2*) and seven *Erysipelotrichaceae* (*Ery1-7*) (10-member community) in **Fig. 7D**, 1 mL of their overnight Mega culture (~1 x 10^7 CFU) were mixed and co-colonized with germ-free mice (n = 3 or 4 per group) via oral gavage (300 µL per mouse). The germ-free mice were maintained on standard chow, and cholic acid sodium salt (5mM for the 10-member community and 0.5 mM for the 3-member community) was supplied in water to facilitate **S122** colonization and ensure both gnotobiotic mice settings have comparable gut bile acid profiles. Successful colonization of **S122** was determined by the Colony-forming unit (CFU) counting (plating on TSAB agar + 10 µg/ml erythromycin and 15 µg/ml thiamphenicol), diagnostic PCR using **S122** specific primers, and LCMS monitoring of CA to DCA conversion of CFU colonies.

For co-colonization of **S122** together with 55 other genetically targetable strains identified in this study, 1 mL of their overnight Mega/RCM/CMM culture (~1 x 10^7 CFU) were mixed and co-colonized germ-free mice (n = 5 per group) via oral gavage (300 µL per mouse). The germ-free mice were maintained on standard chow. Successful colonization of **S122** was determined by LCMS monitoring of CA to DCA conversion, and colonization of **S122** and other strains was confirmed by 16S rRNA sequencing.

For the colonization of **S122** control or its *baiH* mutant in SPF mice, a 300 µL portion of their overnight Mega culture (~1 x 10^7 CFU) were colonized with SPF mice (n = 4 per group) via oral gavage, twice per day for 3 days in a row. The SPF mice were maintained on standard chow (Lab Diet 5053) and water containing thiamphenicol (15 µg/mL) and erythromycin (10 µg/mL). Successful colonization was determined by colony-forming unit (CFU) counting (performed at least weekly, plating on TSAB agar + 10 µg/ml erythromycin and 15 µg/ml thiamphenicol), diagnostic PCR using **S122** specific primers, and LCMS monitoring of CA to DCA conversion of CFU colonies.



Data S1E. Proposed pathway for the 7α-dehydroxylation of cholic acid (CA) to deoxycholic acid (DCA) and the structures of taurine-conjugated and nonconjugated bile acids in this study, related to Figure 4

SUPPLEMENTARY DATA FILE S2

1. Sequences of the nine replication origins. Sequences marked in green are homologous to the plasmid for Gibson assembly.

A. pBP1 (*C. botulinum*)

gaatggcgaatggcgctagcataaaaataagaagcctgcatttgcaggcttcttatttttatggcgcgccgttctgaatccttagctaatggttcaacag
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ggcaagttg

B. pCB102 (*C. butyricum*)

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C. pCD6 (*C. difficile*)

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D. pIM13 (*B. subtilis*)

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E. Cthem-based rep origin (*C. thermocellum*)

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F. pAMβ1-based (*E. faecalis*)

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G. pWV01-based (*L. lactis*)

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H. pMB1 (*B. longum*)

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I. pIP404-based (*C. perfringens*)

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2. Plasmid series pGM-ABCM to pGM-IBCM. One representative GenBank document is shown.

LOCUS pGM-ABCM 6151 bp DNA circular 15-MAY-2019
DEFINITION Shuttle vector pMTL82151. clostron.com.
ACCESSION urn.local...c-agz6tx3
KEYWORDS .
SOURCE null
ORGANISM
.
REFERENCE 1 (bases 1 to 5254)
AUTHORS Heap,J.T., Pennington,O.J., Cartman,S.T. and Minton,N.P.
TITLE A modular system for Clostridium shuttle plasmids
JOURNAL Journal of Microbiological Methods (2009) 78(1) 79-85
PUBMED 19445976
FEATURES Location/Qualifiers
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3. Plasmid series pGM-ABCL to pGM-IBCL. One representative GenBank document is shown.

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ACCESSION .
VERSION .
KEYWORDS pMTL82153_lacza_dcpf-1
SOURCE synthetic DNA construct
ORGANISM synthetic DNA construct
REFERENCE 1 (bases 1 to 9717)
AUTHORS ltt
TITLE Direct Submission
JOURNAL Exported 2021?10?28? from SnapGene 2.3.2
http://www.snapgene.com

FEATURES Location/Qualifiers

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4. Plasmid series pGM-ABCF to pGM-IBCF. One representative GenBank document is shown.

LOCUS pGM-ABCF 10055 bp ds-DNA circular SYN 15-7?-2021
DEFINITION .
ACCESSION .
VERSION .
KEYWORDS pMTL82153_lacza_dcpf-1_gRNA (Ra330_82153_lacza_dcpf-1)
SOURCE synthetic DNA construct
ORGANISM synthetic DNA construct
REFERENCE 1 (bases 1 to 10055)
AUTHORS ltt
TITLE Direct Submission
JOURNAL Exported 2021?10?28? from SnapGene 2.3.2
http://www.snapgene.com
FEATURES Location/Qualifiers
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5. Plasmid series pGM-ABCD to pGM-IBCD. One representative GenBank document is shown.

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ACCESSION .
VERSION .
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SOURCE synthetic DNA construct
ORGANISM synthetic DNA construct
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AUTHORS ltt

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REFERENCE   2  (bases 1 to 9329)
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6. Plasmid series pGM-ACAQ to pGM-lCAQ. One representative GenBank document is shown.

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SOURCE synthetic DNA construct
ORGANISM synthetic DNA construct
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AUTHORS .
TITLE Direct Submission
JOURNAL Exported Sunday, Jul 10, 2016 from SnapGene Viewer 3.1.2
http://www.snapgene.com
REFERENCE 2 (bases 1 to 9812)
AUTHORS .
TITLE Direct Submission
JOURNAL Exported 2021?10?28? from SnapGene 2.3.2
http://www.snapgene.com
FEATURES Location/Qualifiers

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7. Plasmid series pGM-ACBQ to pGM-ICBQ. One representative GenBank document is shown.

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ACCESSION DNA2.0
VERSION .
KEYWORDS pMTL007C-E2-Clostron_82_16S_685_Spec (Ra514+Ra512)
SOURCE synthetic DNA construct
ORGANISM synthetic DNA construct
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AUTHORS .
TITLE Direct Submission
JOURNAL Exported Sunday, Jul 10, 2016 from SnapGene Viewer 3.1.2
http://www.snapgene.com
REFERENCE 2 (bases 1 to 9910)
AUTHORS .
TITLE Direct Submission
JOURNAL Exported 2021?10?28? from SnapGene 2.3.2
http://www.snapgene.com
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8. Plasmid series pGM-NAC$_2$B, NAC$_2$P, NACO. One representative GenBank document is shown.

LOCUS pGM-NAC$_2$B 4204 bp ds-DNA circular SYN 04-4?-2020
DEFINITION synthetic circular DNA.
ACCESSION .
VERSION .
KEYWORDS pExchange_pseudo_Bac_16S_CatP
SOURCE synthetic DNA construct
ORGANISM synthetic DNA construct
REFERENCE 1 (bases 1 to 4204)
AUTHORS .
TITLE Direct Submission
JOURNAL Exported Tuesday, Apr 25, 2017 from SnapGene Viewer 3.3.3
http://www.snapgene.com
REFERENCE 2 (bases 1 to 4204)
AUTHORS .
TITLE Direct Submission
JOURNAL Exported 2021?10?28? from SnapGene 2.3.2
http://www.snapgene.com
COMMENT [DEFINITION]: . [LOCUS]: [ACCESSION]:.
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