LETTER

The evolution of cooperation within the gut microbiota

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Cooperative phenotypes are considered central to the functioning of microbial communities in many contexts, including communication via quorum sensing, biofilm formation, antibiotic resistance, and pathogenesis1–5. The human intestine houses a dense and diverse microbial community critical to health1,2,4–9, yet we know little about cooperation within this important ecosystem. Here we test experimentally for evolved cooperation within the Bacteroidales, the dominant Gram-negative bacteria of the human intestine. We show that during growth on certain dietary polysaccharides, the model member Bacteroides thetaiotaomicron, exhibits only limited cooperation. Although this organism digests these polysaccharides extracellularly, mutants lacking this ability are outcompeted. In contrast, we discovered a dedicated cross-feeding enzyme system in the prominent gut symbiont Bacteroides ovatus, which digests polysaccharide at a cost to itself but at a benefit to another species. Using in vitro systems and gnotobiotic mouse colonization models, we find that extracellular digestion of inulin increases the fitness of B. ovatus owing to reciprocal benefits when it feeds other gut species such as Bacteroides vulgatus. This is a rare example of naturally-evolved cooperation between microbial species. Our study reveals both the complexity and importance of cooperative phenotypes within the mammalian intestinal microbiota.

A major challenge facing the study of host-associated microbiotas is to understand the ecological and evolutionary dynamics that shape these communities2–5,10–13. A key determinant of microbial dynamics is the balance of cooperation and competition both within and between species2,5,14,15. Here we test for the evolution of cooperation within the mammalian microbiota by focusing on the Bacteroidales, the most abundant order of Gram-negative bacteria of the human intestine with species that co-colonize the host at high densities of 108–1011 CFU per gram of feces16,17. Members of this order break down polysaccharides outside of their cell using outer surface glycoside hydrolases6,18, some of which are secreted on outer membrane vesicles5,19. This suggests a significant potential for one cell to cooperatively feed other cells. As extracellular digestion is considered important for growth of Bacteroidales on polysaccharides1,6,8, we focused on this trait as a candidate for cooperative interactions within the gut microbiota and asked whether a bacterium that breaks down a polysaccharide extracellularly receives most, or all20, of the benefits of its efforts.

We first made isogenic mutants in genes responsible for the extracellular digestion of polysaccharides in the well-studied human gut strain, Bacteroides thetaiotaomicron VPI-5482 (refs 1, 8). Specifically, we deleted the genes encoding the outer surface glycoside hydrolases BT3698 of the amylopectin/starch utilization locus and BT1760 of the levan utilization locus (Fig. 1a), required for growth on amylopectin and levan, respectively (Fig. 1b, c, Extended Data Fig. 1a, b). Consistent with previous observations1,8, neither mutant grew with the specific polysaccharide in monoculture (Fig. 1b, c, Extended Data Fig. 1a, b). However, co-culture of ΔBT3698 or ΔBT1760 with wild type in amylopectin or levan increased the fitness of the mutants (Fig. 1d, e, Extended Data Fig. 1c, d). This is consistent with cooperation via public good availability of amylopectin and levan breakdown products.

Figure 1 | Direct and cooperative benefits of polysaccharide digestion by surface glycoside hydrolases (GH). a, Polysaccharide utilization loci of B. thetaiotaomicron (Bt) for amylopectin and levan with the products or properties each gene encodes listed above and colour-coded. SPI or SPII, signal peptidase I or II cleavage site, respectively. b, c, Growth of Bt wild type (WT) and surface GH mutants in media with amylopectin (n = 2, cell culture biological replicates) (b) or levan (n = 2, cell culture biological replicates) (c). See Extended Data Fig. 1 for additional independent experiments. d, e, Growth of Bt WT and surface GH mutants in mono- and co-culture in media with amylopectin (n = 2, cell culture biological replicates) (d) or levan (n = 2, cell culture biological replicates) (e). See Extended Data Fig. 1 for additional independent experiments. In all panels error bars represent standard error; P values derived from two-tailed Student’s t-test.

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Figure 2a, Growth of Bo WT and mutants in inulin defined medium. 
and 
middle panels) and maximal \( A_{600\text{ nm}} \) (bottom panel) of Bo WT or 
\( \Delta \)4502/3 in minimal media with carbon sources as indicated. Each
line represents \( n = 1 \) sample per condition. See Extended Data Fig. 4
for additional independent experiments. In the bottom panel, \( P \) values are
displayed as paired (matched WT and 
\( \Delta \)4502/3 within same experiment) followed by unpaired (all experiments) two-tailed Student’s t-test.

Figure 2 | B. ovatus does not require surface digestion for utilization of inulin. a, Predicted inulin utilization locus of B. ovatus (Bo). Gene designations shown are preceded by BACOVA_. The colour coding of
gene products is as in Fig. 1. b, Thin layer chromatography (TLC) analysis of inulin defined medium incubated for the indicated times with purified
His-tagged 4502, 4503 or 4502 and 4503, or vector control. See Supplementary Information Fig. 1 for uncropped scanned images.
c, Growth of Bo WT and mutants in inulin defined medium. d, TLC
analysis of conditioned media during the growth of Bo WT and mutants in 0.5% and 0.1% inulin. EL, early log; ML, mid log; LL, late log;
St, stationary. 
e, Growth of Bo WT, \( \Delta \)4502/3, \( \Delta \)4504 (susD orthologue) and \( \Delta \)4505 (susC orthologue) mutants in inulin defined medium.
For c, e, each line represents \( n = 1 \) sample per condition. See Extended Data Fig. 2 for additional independent experiments.
In all panels error bars represent standard error; \( P \) values derived from two-tailed Student’s t-test.

One of the key questions in evolutionary biology is how cooperative systems can be evolutionarily stable1,3,5,14,21,22. If certain cells invest in
the production of an enzyme that helps others, what prevents these cells from being outcompeted by cells that consume the breakdown products
without making the enzyme? In the B. thetaiotaomicron amylopectin
and levan polysaccharide utilization systems, while receiving public goods from the wild type benefits the mutant cells (Fig. 1d, e, Extended
Data Fig. 1e), they do not outcompete the wild type. Cells that make
the enzyme receive more benefits than non-producing neighbouring cells. This observation suggests that a cell can utilize the majority of the
polysaccharide that it breaks down, and that these private23 benefits
are central to the evolutionary stability of extracellular polysaccharide
digestions in these systems.

We extended our analysis to another prominent member of the human Bacteroidales known to extensively utilize polysaccharides, Bacteroides ovatus (Bo)24. During growth on inulin, a dietary fructan
known for health-promoting effects25, Bo extracellularly digests and liberates considerable amounts of inulin breakdown products2. The
predicted inulin utilization locus of the Bo type strain ATCC 8483 encodes
two similar outer surface glycoside hydrolases, BACOVA_04502 and
BACOVA_04503 (Fig. 2a), both of which are predicted to target the
\( \beta \)1,2 inulin fructose polymer1,3. Both of these enzymes are required for

Figure 3 | Cost of inulin digestion by surface glycoside hydrolases.
a, Growth yield of Bo WT and mutants on inulin agarose plates. Each line represents \( n = 1 \) sample per condition. See Extended Data Fig. 4
for additional independent experiments. b, Bacteria per gram of faeces of gnotobiotic mice seven days after monocolonization with Bo WT or 
\( \Delta \)4502/3 on a polysaccharide-free diet or supplemented with inulin
(\( n = 5 \) biological replicate mice per condition). c, Growth curves (upper and middle panels) and maximal \( A_{600\text{ nm}} \) (bottom panel) of Bo WT or 
\( \Delta \)4502/3 in minimal media with carbon sources as indicated. Each
line represents \( n = 1 \) sample per condition. See Extended Data Fig. 4
for additional independent experiments. In the bottom panel, \( P \) values are displayed as paired (matched WT and 
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inulin breakdown (Fig. 2b). We therefore predicted that a single mutant of either BACOVA_04502 or BACOVA_04503 would be unable to grow on inulin. Surprisingly, neither of the single deletion mutants (Δ04502 or Δ04503) nor the double mutant (Δ04502/3) demonstrated impaired fitness with inulin as the sole carbohydrate source (Fig. 2c, Extended Data Fig. 2a), even at limiting concentrations (Extended Data Fig. 2a).

Given the importance of extracellular polysaccharide digestion for growth of Bacteroides on numerous polysaccharides1,6,8 (Fig. 1, Extended Data Fig. 1), we predicted that Bo synthesizes other enzymes that breakdown inulin extracellularly, allowing the Bo mutants to grow on this polysaccharide. However, analysis of the growth media of Δ04502, Δ04503 and Δ04502/3 revealed no released inulin breakdown products, demonstrating that BACOVA_04502 and BACOVA_04503 are solely responsible for extracellular digestion of inulin (Fig. 2d, Extended Data Fig. 2b; see Extended Data Fig. 2c for complementation). BACOVA_04504 and BACOVA_04505 encode SusD and SusC orthologues respectively, which are predicted to function in inulin binding and the import of digestion products6,26. Deletion of BACOVA_04504 or BACOVA_04505 resulted in significant impairment of growth on inulin (Fig. 2e, Extended Data Fig. 3a; see Extended Data Fig. 3b for complementation). Growth of Δ04502, Δ04503 and Δ04502/3 in limiting concentrations of inulin revealed depletion of inulin (Fig. 2d, right panel; Extended Data Fig. 2b). Together, these data demonstrate that surface enzymes 04502 and 04503 are not needed for Bo to utilize inulin, and suggest that inulin is directly imported via 04504-04505 without prior extracellular digestion.

Why would Bo synthesize surface/secreted enzymes that potently digest inulin outside of the cell if not necessary for its growth on the polysaccharide? A key evolutionary explanation for the release of secreted products by microbes is that they feed clonemates in a manner that is beneficial at the level of the clonal group2,5,11,14,15. We hypothesized that the importance of extracellular digestion may be realized during spatially structured growth on plates where not all cells are in direct contact with the polysaccharide. However, mutant bacteria showed no significant differences in growth yield compared to wild type on defined inulin plates (Fig. 3a; Extended Data Fig. 4a). In addition, these enzymes were not required for optimal growth in vivo as wild type and Δ04502/3 showed equal colonization levels in gnotobiotic mice (n = 3 cell culture biological replicates) with Bo WT and Δ04502/3. Polysaccharide-free diet was supplemented with inulin at day 7. Bo was introduced at day 14. A Fisher exact test comparing the frequency of Bo WT and Δ04502/3 pre- (day 14) and post- (day 18) colonization with Bo was significant with a P value of 0.0001 for each individual mouse. Bo CFU in faeces were maximal after the switch to inulin diet and addition of Bo changed the abundance of Bo WT compared to Δ04502/3 but not total CFU of Bo. See Extended Data Fig. 9 for additional independent experiments. In all panels error bars represent standard error; P values displayed are derived from two-tailed Student’s t-test.

Figure 4 | Interspecies cooperation mediated by surface digestion of inulin is stabilized by reciprocal benefits. a, Germ-free mice were mono-colonized with Bo WT or Δ04502/3 and maintained on a polysaccharide-free diet supplemented with inulin and housed for 2 weeks. Mice were then gavaged with the caecal contents of conventionally raised mice. At day 5 post gavage, caecal contents were plated for enumeration of Bo (n = 5 biological replicate mice per group). b, Enumeration of Bo in monotype or co-culture with Bo WT or Δ04502 on defined inulin plates. *P = 0.001 for number of Bo in Bo/Bo WT co-culture vs Bo alone; †P = 0.001 for number of Bo in Bo/Bo WT co-culture vs Bo/Δ04502 co-culture; ‡P = 0.003 for number of Bo in Bo/Δ04502 co-culture vs Bo alone (n = 2, cell culture biological replicates). See Extended Data Figs 7 and 9 for additional independent experiments. c, (left) Photos of patches of Bo plated at varying distances around Bo WT or Bo Δ04502/3 or alone on inulin agarose plates. (right) Enumeration of Bo after five days of culture plated at the same distance to Bo WT or Bo Δ04502/3 or alone on inulin plates. See Extended Data Fig. 9 for additional independent experiments. d, Enumeration of Bo WT or Δ04502 in monotype or co-culture with Bo on defined inulin plates. P values correlate to colour of line/genotype used for statistical analysis. P value indicates comparison of monotype and co-culture for the given condition at the time-point indicated. The benefit Bo receives from Bv is most robust when starting with fewer Bo. Depicted are starting CFU of ~10⁶ Bo (n = 3, cell culture biological replicates at day 1, 2, n = 2, biological replicates at day 4). See Extended Data Fig. 7 for additional independent experiments and Extended Data Fig. 7b for starting CFU of ~10⁷ Bo. e, Ratios of wild type and Δ04502/3 at the start and day 2 of culture when co-plated with or without Bo on inulin plates (n = 3, cell culture biological replicates). See Extended Data Fig. 9 for additional independent experiments. f, Ratios of WT and Δ04502/3 in the inoculum (day 0) and in faeces at various time points (days 4, 7, 11, 14, 18) post co-colonization of gnotobiotic mice (n = 3 cell culture biological replicates) with Bo WT and Δ04502/3. Polysaccharide-free diet was supplemented with inulin at day 7. Bo was introduced at day 14. A Fisher exact test comparing the frequency of Bo WT and Δ04502/3 pre- (day 14) and post- (day 18) colonization with Bo was significant with a P value of 0.0001 for each individual mouse. Bo CFU in faeces were maximal after the switch to inulin diet and addition of Bo changed the abundance of Bo WT compared to Δ04502/3 but not total CFU of Bo. See Extended Data Fig. 9 for additional independent experiments. In all panels error bars represent standard error; P values displayed are derived from two-tailed Student’s t-test.
mutant does not occur under limiting inulin concentrations (Extended Data Fig. Sd,e). Furthermore, Bo preferentially consumes longer inulin digestion products over shorter oligomers and fructose (Fig. 2d, right panel, Extended Data Fig. 2b, right panel). Together, these data suggest that undigested inulin is the preferred substrate of Bo, and that extracellular digestion by Δ04502/3 under certain conditions is costly for fitness.

We found no evidence that extracellular digestion of inulin by Bo evolved for cooperation with clonemates. Therefore, we speculated that this trait might have evolved for cooperation with other species in the gut. We first sought evidence of cooperation in the setting of a natural gut ecosystem. Germ-free mice were fed inulin and colonized with either Bo wild type or Δ04502/3 followed by the introduction of the caecal microbiota of conventionally raised mice. Bo wild type and Δ04502/3 equally colonized mice before the introduction of the microbiota (Extended Data Fig. 6a), but Bo wild type received a significant fitness benefit compared to Δ04502/3 in the context of a complex microbiota (Fig. 4a). These data suggested that, although not required for Bo to utilize inulin, 04502 and 04503 provide a benefit to Bo only realized in a community setting. The conditions for the evolution of cooperation between species are much more restrictive than those within a clone. In particular, theory predicts that costly interspecies cooperation will only be stabilized if there are reciprocal feedback benefits, such as a plant providing nectar for an insect that pollinates it1,2,12. From this experiment, we identified two dominant mouse microbiota Bacteroides strains that thrived on Bo-derived inulin breakdown products (Extended Data Fig. 6b), with delayed growth on inulin (Extended Data Fig. 6b). These data suggest that cross-feeding Bacteroides members may provide reciprocal benefits to wild-type Bo in the mammalian gut.

To test experimentally for reciprocity and benefits of inulin digestion between species, we used an inulin co-culture system with Bo wild type or Δ04502/3 and Bacteroides vulgatus ATCC 8482 (Bv), which is commonly found together with Bo at high densities in humans1,6,17 and thrives on inulin breakdown products but cannot use inulin1,3. Co-culture and plating experiments with Bo wild type increased the fitness of Bv compared to that with Δ04502/3 (Fig. 4b, c and Extended Data Figs 7a, 9a); however, Bo is able to persist better with Δ04502/3 than when alone (Fig. 4b, Extended Data Fig. 7a), owing to a small (<2,000 Da) secreted molecule(s) that contributes to the survival of Bv (Extended Data Fig. 8a, b). This 04502/3-independent survival is not mediated by a universal factor made by Bacteroides during growth on inulin nor Bo-derived short-chain fatty acids (Extended Data Fig. 8a, c, d). Thus, there are multiple mechanisms by which Bo helps Bv (Fig. 4b, Extended Data Fig. 7a), the greatest being cross-feeding mediated by 04502/3.

We next addressed the question of whether Bo receives reciprocal benefits from Bv. Co-culture of Bv with Bo on plates increased the fitness of Bo wild type and Bo Δ04502/3 (Fig. 4d, Extended Data Fig. 7b), but did not increase the fitness of B. fragilis (Extended Data Fig. 7c). If inulin breakdown can be costly, and Bo receives benefits from Bv irrespective of whether inulin is broken down and fed to Bv, natural selection is expected to favour the loss of the genes encoding the secreted inulin glycoside hydrolases1,22. However, our pairwise experiments would not reveal the possibility that Bo wild type receives more reciprocal benefits from Bv when in direct competition with the non-cross-feeding mutant1,2,12. Therefore, we co- and tri-cultured these strains on plates and compared the yields of the two Bo strains (wild type and Δ04502/3) with or without Bv. Addition of Bv leads to an increased proportion of Bo wild type compared to the Δ04502/3 mutant (Fig. 4e, Extended Data Fig. 9b). We extended these studies to a gnotobiotic mouse colonization model. Bo wild type had no advantage in direct competition with Δ04502/3 on a polysaccharide-free diet or when inulin is added (Fig. 4f). However, introduction of Bv increased the fitness of the Bo wild type relative to the mutant (Fig. 4f, Extended Data Fig. 9c, d). Together, these data suggest that extracellular breakdown of inulin increases the fitness of Bo via reciprocal benefits from another species. These findings are consistent with the evolution of cooperation between species within the gut microbiota.

We find evidence of distinct forms of cooperativity within the Bacteroidales of the human intestinal microbiota (Extended Data Fig. 10). For B. thetaiotaomicron, amylopectin and levan digestion provide mostly private benefits and modest social benefits to other cells. By contrast, Bo releases large amounts of inulin digestion products via a pair of dedicated cross-feeding secreted enzymes unnecessary for its use of inulin. These enzymes allow for cooperation with cross-fed species, which provide benefits in return. Potential mechanisms by which Bv may provide return benefits to Bo include detoxification of inhibitory substances, or production of a depleted or growth promoting factor, the latter supported by early growth benefits to Bo via Bv secreted factors (Extended Data Fig. 9e).

Understanding whether microbial communities are formally cooperative is central to predicting their evolutionary and ecological stability. Cooperative systems can be productive, but are prone to instabilities on both ecological and evolutionary timescales that can undermine them1,4,15,21,22. The ability of one species to utilize the waste product of another is prevalent, but waste production alone does not signify cooperative evolution. As opposed to waste product utilization or exploitive interactions28–30, there are few well-documented cases of evolved cooperation between microbial species where one species evolves to help another14. We have found evidence of strong eco-evolutionary interactions within the microbiota that are likely to be central to the functioning of these complex communities.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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

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Bacterial strains and media. Bacteroidales type strains used in this study are Bo ATCC 8483, B. thetaiotaomicron VPI 5482 Bv ATCC 8482, and B. fragilis NCTC 9343. Bacteria were grown in media formulation as previously described. For growth in defined media, bacteria were inoculated from brain heart infusion plates containing haemin and vitamin K (BHIS) plates into basal medium (BS), cultured overnight to stationary phase, then diluted 1:10 in fresh BS and grown to mid log. At mid log, bacteria were pelleted by centrifugation and washed twice with sterile phosphate buffered saline (PBS) and then inoculated in defined media. Carbohydrates used to supplement defined media include fructose (F2543, Sigma), fructose oligosaccharides (FOS; OraftiP95, Bienen-Orafti group), levans (L8467, Sigma), amylose (10120, Sigma), and inulin (OraftiHP, Bienen-Orafti group). Levans and amylopectins were autoclaved at 1% w/v in H2O and dialysed using 3.5 kDa MW membranes (Slide-A-Lyzer Dialysis Cassettes, ThermoScientific). Short chain fatty acids acetate, propionate and succinate were purchased from Sigma. Stock solutions of 2 mM were pH neutralized to pH 7.2−7.3 with 10% NaOH. All cultures were grown at 37 °C under anaerobic conditions. Bacterial growth was quantified by absorbance (A600nm) using 200 μl of bacterial culture in 96-well flat-bottom microtitre plates using a Powerwave spectrophotometer (Biotek). Murine gut Bacteroidales from the caecal preparations used in for the colonization experiments were grown on BHIS plates. Resulting colonies were tested for growth in inulin minimal medium or inulin breakdown products from the conditioned media of Bo grown in inulin, containing inulin breakdown products as previously described.

Creation of Bacteroides mutants. Deletion mutants were created whereby the genes encoding BT3698 or BT1760 in Bacteroides thetaiotaomicron VPI 5482, BACOV A_04502 in Bo ATCC 8483 BACOVA 04503, BACOVA 04502/3, BACOVA 04504, or BACOVA 04505 in Bo ATCC 8482 were removed. DNA segments upstream and downstream of the region to be deleted were PCR amplified using the primers outlined in Supplementary Table 1. PCR products were digested with BamHI, EcoRI and/or MluI engineered into the primers (Supplementary Information Table 1) and cloned by three-way ligation into the appropriate site of pNZR6 (ref. 31). The resulting plasmids were conjugally transferred into the Bacteroides strain as indicated using helper plasmid R751 and cointegrates were selected by erythromycin resistance. Cross overs were screened by PCR for the mutant genotype.

Cloning of PUL genes for expression in deletion mutants. BACOVA 04502, BACOVA 04503, BACOVA 04502/3, BACOVA 04504, or BACOVA 04505 or BACOVA 04504/5 genes were PCR amplified using the primers listed in Supplementary Table 1. The PCR products were digested and ligated into the BamHI or KpnI site of the Bacteroides expression vector pFD3403. Plasmids containing the correct orientation of the insert in relation to the vector-borne promoter were introduced into mutant Bacteroides strains by mobilization from E. coli using helper plasmid RK231.

Mono-, co- and tri- culture experiments. For bacterial mono and co-culture experiments in defined liquid media, bacteria were grown as indicated for monoculture before addition to the defined media. Sterile magnetic stir bars were added to culture tubes within a rack placed on a stir plate within the anaerobic chamber. For conditioned media experiments, Bo WT, Bo Δ04502, Bo Δ04502/3, Bv or Bf were grown to early log in inulin defined media or 0.125% fructose defined media (for Extended Data Fig 9e), conditioned media were collected, filter sterilized, and incubated at 37 °C for 72h. Conditioned media was replenished with defined media without additional carbohydrate and used for cultivation of Bv. Bo Δ04502/3 conditioned media was dialysed in defined media without carbohydrate using 2 kDa MW membranes (Slide-A-Lyzer Dialysis Cassettes, ThermoScientific). For monoculture of Bo WT and mutants on solid agarose, 4 μl of the indicated concentration of bacteria were spotted onto minimal inulin agar plates. At the indicated time points, plates were cut out, diluted and plated onto BHIS for CFU enumeration. For co-culturing experiments, 105 Bo (WT or mutant) or Bv were co-plated with 105 Bf or a control volume of PBS. Four μl were then dotted on to defined inulin agar plates. At the indicated time points, the dotted patches were cut from the agarose plates and resuspended in PBS, diluted and plated to BHIS for enumeration. Quantification and differentiation of wild type and isogenic glycoside hydrolase or polysaccharide lyase mutant was performed by plating dilutions of mixed liquid culture or the cut-out patch on agarose plates onto BHIS, followed by picking ~100 colonies and determining wild type or mutant genotype by PCR using primers listed in Supplementary Table 1. For genotypic screening of B. thetaiotaomicron wild type and B. thetaiotaomicron Δ04508, two sets of primers were used (Supplementary Table 1). For Bo/Bv co-culture in Fig 4b, c and Extended Data Fig 7, Bo Δ03533 (WT) and Bo Δ03533 Δ04502 (Δ04502) arginine auxotrophic mutants were used in co-culture with Bv on minimal inulin agar plates supplemented with 5μg ml−1 of arginine (Sigma) which does not impair or limit growth as compared to wild type. Colonies on BHIS plates were replica plated onto defined glucose defined plates, which support the growth of Bv but not Bo Δ03533 or Bo Δ03533 Δ04502.

Thin-layer chromatography. Thin-layer chromatography (TLC) was employed to specifically detect carbohydrates as previously described. Standards for TLC included glucose (G7528, Sigma), fructose (F2543, Sigma), fructose oligosaccharides (FOS; OraftiP95, Bienen-Orafti group) and inulin (OraftiHP, Bienen-Orafti group). See Supplementary Information Fig 1 for uncorrected TLCs.

Gas chromatographic analysis of culture media. Chromatographic analysis was carried out using a Shimadzu GC14-A system with a flame ionization detector (FID) (Shimadzu Corp, Kyoto, Japan). A volatile acid mix containing 10 mM of acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, caproic, and heptanoic acids was used (Matreya, Pleasant Gap PA). A non-volatile acid mix containing 10 mM of pyruvic and lactic acid and 5 mM of oxaloacetic, oxalic, methyl malonic, malonic, fumaric, and succinic acid was used (Matreya, Pleasant Gap PA).

Cloning, purification, and enzymatic analysis of BACCOVA 04502-3. To obtain purified BACCOVA 04502 and BACCOVA 04503 proteins, these genes were cloned individually into the BamHI site of pET16b (Novagen) using the primers listed in Supplementary Table 1. The constructs were designed so that the His-tag encoded by pET16b replaced the SpI signal sequence of these proteins allowing for their solubility. The recombinant plasmids were transformed into E. coli BL21 (DE3), grown to an A600nm of 0.6−0.7, and expression of the recombinant gene was induced by the addition of 0.4 mM IPTG for an additional 4 h. The His-tagged proteins were isolated essentially as described using Ni2+-NTA TALON paramagnetic beads. For enzymatic analysis (Fig 2a) the proteins were added to inulin media in their magnetic bead-bound form. This allows for easy removal of the enzymes following digestion. As a control, the beads resulting from the same procedure performed with E. coli BL21 (DE3) containing only the vector (pET16b) were used. For bo growth assays (Fig 3d), 50 μl of beads (25 μl containing His-04502 and 25 μl containing His-04503) or equivalent volume of Dynabead buffer (for undigested inulin) were added to inulin defined medium. After 24h at 37 °C, the beads containing the enzyme were removed with a magnet, and the media (digested or undigested inulin) was used to culture Bo WT and Δ04502.

Gnotobiotic mouse experiments. All animal experiments were approved by the Harvard Medical School IACUC. Swiss Webster germ-free male mice (6−10 weeks old) were purchased from the Charles River. Mice were housed in individually ventilated cages (IVC) with filter ventilated inserts. IVCs were exchanged every 7 days. Mice were placed on a diet of polysaccharide-free special chow (65% w/v glucose, protein-free, supplemented with all essential amino acids except arginine; BioServe). Arginine at 50 μg ml−1 was supplemented in all drinking water. As indicated, mice were given 1% inulin (w/v) in sterile drinking water. Mice were inoculated with the indicated bacteria (or their corresponding Δ strain) by applying ~107 live bacteria (grown to mid log) onto mouse fur. Dilutions of faeces at various time points following inoculation were plated to BHIS plates and genotyped as above (Fig 4f). Predetermined exclusion criteria for gnotobiotic experiments contamination as determined by either the presence of colonies with distinct morphology on anaerobic plates than Bo or Bv or colonies present at >10² CFU ml−1 (limit of detection) under aerobic conditions.

For gavage of Bo WT or Δ04502/3 monoclonized mice with the caecal content of conventionalized raised mice, two ~ 8-week-old male Swiss Webster mice purchased from Taconic and housed in a specific pathogen-free facility were used. Littermates were randomly allocated for different gnotobiotic experimental arms. Experiments were conducted in either sterile Optima cages (Figs 3b, 4a, Extended Data Fig 9d) or gnotobiotic isolators (Fig. 4f). When appropriate, animals were housed in cages of 10−20 mice to increase environmental challenges. Mice were housed in cages of 8−10 mice per cage. At time of killing, caecal contents were collected and plated to defined inulin plates by which Bo was enumerated based on distinct colony morphology of Bo on inulin agar plates that were not present in conventionally raised caecal population.
Statistical analysis. The experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment. No statistical methods were used to predetermine sample size. Replicate experiments are shown in Extended Data. All P values are derived from Student’s t-test except as indicated in Fig. 4f and Extended Data Fig. 9c where Fisher’s exact test was performed. Statistical significance of variance reported as indicated per experiment in figure legends. All centre values are mean. Error bars are standard error of the mean.

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Extended Data Figure 1 | Limited cooperation during polysaccharide utilization by *B. thetaiotaomicron*. a–d, Independent experiments for Fig. 1b–e. a, b, Upper panels $n = 2$ biological replicates, lower panels $n = 1$; c, d, $n = 2$ biological replicates. e, TLC analysis of conditioned media from *B. thetaiotaomicron* grown in amylopectin (left panel) or levan (right panel) minimal media. EL, early log; ML, late log; LL, late log; Stat, stationary phase; Glu, glucose; Fru, fructose; Suc, sucrose. See Supplementary Information Fig. 1 for uncropped scanned images. In all panels, error bars represent standard error; $P$ values derived from two-tailed Student’s $t$-test.
Extended Data Figure 2 | Bo 04502 and 04503 mutants grow equivalently to wild type on limiting concentrations of inulin and do not require surface digestion for utilization of inulin. **a**, Growth of Bo WT, Δ04502, Δ04503 and Δ04502/3 mutants in varying concentrations of inulin as indicated. Biological replicates of each condition are plotted as individual lines (n = 2 cell culture biological replicates). Upper and lower panels are independent experiments. **b**, Independent experiments for Fig. 2d. EL, early log; ML, mid log; LL, late log; St, stationary phase. **c**, Complementation of Bo Δ04502 and Δ04502/3 mutants with the respective genes in trans. TLC analysis of conditioned media from Bo Δ04502/3 (left panel) complemented in trans with BACOVA_04502, BACOVA_04503, BACOVA_04502/3 or vector alone (pFD340) and Bo Δ04502 (right panel) with BACOVA_04502 or vector alone grown in defined inulin media. See Supplementary Information Fig. 1 for uncropped scanned images. S, stationary phase.
Extended Data Figure 3 | *SusC* and *SusD* homologues BACOA_04505 and BACOA_04505 are required for inulin utilization. a, Independent experiments for Fig. 2e. Left panel *n* = 2 biological replicates, right panel each line represents *n* = 1 sample per condition. b, Complementation of *Bo Δ04504*, *Bo Δ04505* and *Δ04504/5* mutants with the genes *in trans*. Growth of *Bo Δ04504*, *Bo Δ04505*, *Bo Δ04504/5* with BACOA_04504, BACOA_04505, BACOA_04504/5 or vector alone (pFD340) *in trans* in defined inulin media. Each line represents *n* = 1 sample per condition. In all panels, error bars represent standard error; *P* values derived from two-tailed Student’s *t*-test.
Extended Data Figure 4 | Costs of extracellular inulin digestion by *B. ovatus*. **a**, Independent experiments for Fig. 3a, c. *n* = 3 biological replicates at day 1, 2; *n* = 2, biological replicates at day 4 (a). In upper and upper middle panels biological replicates of each condition are plotted as individual lines (*n* = 2 cell culture biological replicates); in lower middle and lower panels each line represents *n* = 1 sample per condition (b).

c. TLC analysis of conditioned media from *Bo WT* and *Bo Δ04502/3* cultured in 0.5% inulin with trace (0.06%) amounts of fructose. St, stationary phase. See Supplementary Information Fig. 1 for uncropped scanned images. In all panels, error bars represent standard error; *P* values derived from two-tailed Student’s *t*-test.
Extended Data Figure 5 | Preferential utilization of undigested inulin by *B. ovatus* and costs of inulin digestion by 04502/3. a, Time to mid-log (estimated at 50% maximal OD (OD50) by linear regression analysis) of Fig. 3d. b, Additional independent experiments for Fig. 3d. Each line represents *n* = 1 sample per condition. c, Competition of *Bo* WT and *Bo* Δ04502/3 co-cultured in 0.5% inulin with trace (0.06%) amounts of fructose. *n* = 2 cell culture biological replicates; d, e, Growth (d) and TLC analysis of conditioned media (e) of *Bo* WT and *Bo* Δ04502/3 cultured in 0.1% inulin with trace (0.06%) amounts of fructose. See Supplementary Information Fig. 1 for uncropped scanned images. For d, each panel is an independent experiment. Each condition is plotted as individual lines in each panel (*n* = 2 cell culture biological replicates). In all panels, error bars represent standard error; *P* values derived from two-tailed Student’s *t*-test.
Extended Data Figure 6 | A complex mouse microbiota differentially affects *B. ovatus* WT and Δ04502/3 pre-colonized gnotobiotic mice and analysis of cross feeding of the predominant murine Bacteroidales 
of the murine gut microbiota. a, Germ-free mice were monocolonized 
with *Bo* WT or Δ04502/3 and maintained on a diet supplemented with 
inulin as the sole polysaccharide and housed under gnotobiatic conditions for 2 weeks. Bacteria were enumerated from faeces before gavage with 
intestinal microbiota of conventionally raised mice (*n = 5* mice, cell culture 
biological replicates). b, Growth of two dominant mouse microbiota 
Bacteroidales strains (*Parabacteroides goldsteinii* and a strain with 96% 16S rRNA gene identity to *B. thetaiotaomicron*) with inulin breakdown 
products derived from the conditioned media of *B. ovatus* grown in inulin (all inulin had been digested) or undigested inulin minimal media. Each 
data point is a different isolate of the indicated species from the caeca of the conventionally raised mice used for gavage (*n = 11* isolates for upper 
panel, *n = 6* for lower panel). In all panels, error bars represent standard error.
Extended Data Figure 7 | The *B. ovatus*, but not *B. fragilis* benefits from *B. vulgatus* in co-culture in inulin. a, b, Independent experiments for Fig. 4b, d. The left panel corresponds to starting culture with \( \sim 10^7 \) CFU Bo corresponding to starting culture of \( \sim 10^6 \) CFU Bo in Fig. 4d. The two right panels are a duplicate pair of experiments of starting CFU of \( 10^6 \) and \( 10^7 \). In each panel \( n = 2 \) biological replicates. c, Enumeration of *B. fragilis* in monoculture or co-culture with *Bv* on defined inulin plates, \( n = 3 \) cell culture biological replicates. Letters in parentheses refer to values correlating to colour of line used for statistical analysis. In a, for example, \( p \) (g,r) refers to comparison of values of green (g) and red (r) values at the time-point indicated. In b, colour of \( P \) value indicates comparison of monoculture and co-culture for the given condition at the time-point indicated. For all panels, error bars represent standard error; \( P \) values derived from two-tailed Student’s *t*-test.
Extended Data Figure 8 | Secreted factors from Bo and isogenic mutants, but not B. fragilis (Bf), support Bv survival. a, Growth of Bv in conditioned media from Bo WT, Δ04502, Δ04502/3 or Bf grown in defined media with inulin as the sole carbohydrate and in inulin media. End time-point corresponded to peak growth of Bv in conditioned media derived from Bo WT. B. fragilis, which utilizes inulin1 but similar to Bo Δ04502/3 does not liberate inulin breakdown products3, does not support the survival of Bv during co-culture. Left and right panels are independent experiments. Left panel; start, n = 4 biological replicates; end, n = 2 biological replicates. Right panel; t₀, n = 4 cell culture biological replicates; t13 and 24, n = 2 biological replicates. b, Growth of Bv in dialysed (2 kDa MW membrane) or undialysed conditioned media from Bo Δ04502/3 grown in inulin, n = 2 cell culture biological replicates. c, Gas chromatographic analysis of acetate, propionate and succinate in conditioned media during growth of Bo WT and Δ04502/3 in defined media with inulin as the sole carbohydrate. Other volatile and non-volatile substances (as listed in Methods) were undetectable, n = 2 cell culture biological replicates, except Δ04502/3 stationary phase, n = 1. d, Growth of Bv in defined medium with inulin as the sole carbohydrate with or without addition with 15 mM of acetate, propionate or succinate, t₀, n = 4 biological replicates; t = 13 and 24 h, n = 2 biological replicates. CM, conditioned media; UD, undetected. For all panels, error bars represent standard error; P values derived from two-tailed Student’s t-test.
Extended Data Figure 9 | Spatial aspects of mutualism between Bo and Bv via cross feeding and interspecies cooperation in vivo via 04502/3. 

a, Independent experiments for Fig. 4c, n = 4 biological replicates. 
b, Independent experiments for Fig. 4e, n = 3 cell culture biological replicates. 
c, Scatter plot of experiment in Fig. 4f, n = 3 cell culture biological replicates. 
d, Ratios of wild type and ∆04502/3 in faeces 21 days after co-colonization of three germ-free mice (n = 3 mice biological replicates) on a diet of inulin as the sole dietary polysaccharide (pre-Bv) and then four days after introduction of Bv (post-Bv). Each panel shows the ratio pre- and post- Bv of an individual mouse. P values are Fisher exact test comparing the frequency of Bo WT and ∆04502/3 pre- and post- colonization with Bv for each individual mouse. At day 21, all mice were colonized with a higher ratio of the mutant (ranging to 86% in the mouse depicted in the lowest panel), with each mouse showing a statistically significant increase in the proportion of the wild type after introduction of Bv. 
e, Growth of B. ovatus in 0.5% inulin (upper panel) or fructose (lower panel) in minimal media to which filter sterilized conditioned media from early log, A600 nm matched growth of Bv or Bo in 0.125% fructose minimal media or fresh 0.125% fructose minimal media control was added at 1:1 ratio. In e, numbers refer to P values (*P < 0.05, **P < 0.01, ***P < 0.001) of comparison of values of green (g), red (r) or black (b) values by unpaired, two-tailed student t-test at the time-point indicated, n = 2 cell culture biological replicates. For all panels, error bars represent standard error; for all panels except d, P values derived from two-tailed Student’s t-test.
Extended Data Figure 10 | Schematic of forms of cooperativity via polysaccharide digestion among Bacteroidales. a. Limited cooperation. Privatization of extracellularly digested public goods by the individual performing the digestion leads to greater individual (thick arrow) than shared benefits (thin arrow) as seen in Bt during growth on levan and amylpectin. b. Cooperation between species is seen between Bo and Bv during growth on inulin. Surface digestion of inulin by Bo creates breakdown products that it does not need to grow on inulin. Rather, inulin breakdown represents a dedicated cross-feeding system that provides benefits to Bv, with reciprocal fitness benefits to Bo.