Characterization of inositol lipid metabolism in gut-associated Bacteroidetes

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Inositol lipids are ubiquitous in eukaryotes and have finely tuned roles in cellular signalling and membrane homeostasis. In bacteria, however, inositol lipid production is relatively rare. Recently, the prominent human gut bacterium Bacteroides thetaiotaomicron (BT) was reported to produce inositol lipids and sphingolipids, but the pathways remain ambiguous and their prevalence unclear. Here, using genomic and biochemical approaches, we investigated the gene cluster for inositol lipid synthesis in BT using a previously undescribed strain with inducible control of sphingolipid synthesis. We characterized the biosynthetic pathway from myo-inositol-phosphate (MIP) synthesis to phosphoinositol dihydroceramide, determined the crystal structure of the recombinant BT MIP synthase enzyme and identified the phosphatase responsible for the conversion of bacterially-derived phosphatidylinositol phosphate (PIP-DAG) to phosphatidylinositol (PI-DAG). In vitro, loss of inositol lipid production altered BT capsule expression and antimicrobial peptide resistance. In vivo, loss of inositol lipids decreased bacterial fitness in a gnotobiotic mouse model. We identified a second putative, previously undescribed pathway for bacterial PI-DAG synthesis without a PIP-DAG intermediate, common in Prevotella. Our results indicate that inositol sphingolipid production is widespread in host-associated Bacteroidetes and has implications for symbiosis.

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Here we combine genomic and biochemical approaches to functionally characterize the predicted inositol lipid metabolism gene cluster in BT, from the initial synthesis of myo-inositol-phosphate (MIP) to its addition as a headgroup to glycerophospholipids and SLs. We identify inositol as a component of the cell capsule, and roles for inositol lipids in antimicrobial peptide resistance and bacterial fitness in a mammalian host. Finally, we describe a novel putative alternative gene cluster common in Prevotella, revealing an extensive capacity for inositol lipid synthesis among gut-associated Bacteroidetes.

**Results**

First, we identified genes responsible for BT inositol lipid metabolism (Fig. 1a). We identified BT_1522 as having high homology to the yeast enzyme inositol phosphorylceramide synthase (IPC synthase, also known as AUR1) that catalyses the attachment of the phosphorylinositol group onto ceramide (query cover 50%, e-value $1 \times 10^{-15}$, percent identity 26%). Thus, we hypothesized that BT_1522 is involved in the synthesis of phosphoinositol dihydroceramide (PI-DHC).

BT_1522 and its gene cluster (Fig. 1b) were previously predicted to be involved in inositol lipid metabolism. Adjacent predicted genes in the cluster encode BT_1523 (annotated as a CDP-diacylglycerol-inositol 3-phosphatidyltransferase), BT_1524 (hypothetical protein), BT_1525 (annotated as phosphatidylinositol-phosphate synthase, PpgA) and BT_1526 (myo-inositol phosphate synthase, MIPS).

We constructed a BT strain with inducible control of the first enzyme in the de novo SL synthesis pathway, serine palmitoyltransferase (SPT; BT_0870; Fig. 1a). This inducible SPT (iSPT) strain enables
Fig. 2 | BT produces inositol phospholipids and sphingolipids. a, TLC of five standards: PI-DAG, 16:0 phosphatidylinositol; CPE, ceramide phosphoethanolamine; PE-DAG, egg yolk phosphatidylethanolamine; Sa, d18:0 sphinganine; Cer, d18:1/18:0 ceramide (left); six standard (non-acidic) lipid extracts from the iSPT BT strain (used as a background for knockout generation) at 0, 0.2, 1, 5 and 100 ng ml\(^{-1}\) aTC induction of SPT, and WT BT VPI-5482 (middle); and standard lipid extraction from ΔBT\(_{1522}\), ΔBT\(_{1523}\), ΔBT\(_{1525}\) and ΔBT\(_{1526}\) knockout strains in the iSPT background at 100 ng ml\(^{-1}\) aTC induction of SPT (right). b, TLC of standards: PI-DAG, PE-DAG as in a (left), plus PIP-DAG, 18:1 PI(3)P (left); PIP-DAG lipid extractions of iSPT strains at 0 and 100 ng ml\(^{-1}\) aTC followed by iSPTΔBT\(_{1522}\), iSPTΔBT\(_{1523}\), iSPTΔBT\(_{1525}\) and iSPTΔBT\(_{1526}\) (middle); and each of their respective complementations at 100 ng ml\(^{-1}\) aTC induction of SPT (right). c, Predicted structures and ion chromatograms demonstrating detection of inositol lipids and sphingolipids in iSPT, iSPTΔBT\(_{1522}\), iSPTΔBT\(_{1523}\), iSPTΔBT\(_{1525}\) and iSPTΔBT\(_{1526}\) at 100 ng ml\(^{-1}\) aTC induction. Branching patterns of DHC-based lipids are predicted and not confirmed.
precise control over SL synthesis to produce both PI-DAG and PI-DHC, or solely PI-DAG. As expected, in the absence of SPT induction, we detected no SLs by thin-layer chromatography (TLC) analysis (Fig. 2). Gradual SPT induction led to tunable increases in SL levels, approximating wild-type (WT) SL levels at full induction (Fig. 2a), with SLs comprising ~50% of lipids (47 ± 7%, n = 6). Detected SLs included PI-DHC and phosphoethanolamine dihydroceramide (PE-DHC) (Fig. 2c).

Functional characterization of the inositol lipid cluster. To uncover the function of each enzyme in the putative inositol lipid metabolism pathway, we knocked out individual genes (BT_1522 to BT_1526) in the iSPT background by scarless deletion21 (denoted iSPTΔBT_1522 to iSPTΔBT_1526). Each gene was also knocked out in the WT (Δtdk) background (indicated by for example, ΔBT_1522). We examined the lipid content of the resulting strains (SL synthesis fully induced, unless otherwise stated) using TLC and high performance liquid chromatography mass spectrometry (HPLC–MS) (see Supplementary Information for more detailed lipid structure analysis). Consistent with the predicted role for BT_1522 as a PI-DHC synthase, the iSPTΔBT_1522 strain failed to produce PI-DHC, but production of PI-DAG and non-inositol SLs, including PE-DHC, was unaltered (Fig. 2a–c, and Extended Data Figs. 1 and 2). Similarly, the iSPTΔBT_1526 strain (lacking the predicted MIPS) failed to produce both PI-DAG and PI-DHC, in accordance with the loss of the myo-inositol-phosphate substrate. Interestingly, both the iSPTΔBT_1523 and iSPTΔBT_1525 strains also failed to produce PI-DAG and PI-DHC (Fig. 2). The synthesis of other glycerophospholipids was unaffected in the iSPTΔBT_1525 strain, an observation in disagreement with the annotated function of BT_1525 as a PgpA22. We hypothesized that BT may use a two-step process to synthesize PI-DAG, similarly to Mycobacteria, using a PIP-DAG intermediate18. Accordingly, comparison of the functional protein motifs in BT_1523 and BT_1525 with those in the charac-

Fig. 3 | BT_1526 produces myo-inositol-phosphate in vitro. a, Proposed mechanism for the MIPS-catalysed NAD-dependent/redox-neutral conversion of G6P to MIP. b, Molybdenum blue assay for detection of MIP. Kinetic analysis of recombinant BT_1526 MIPS using G6P as substrate. c, The crystal structure of BT_1526 MIPS: (i) The monomer subunit, (ii) the tetramer, with cartoon representations to illustrate relative rotations of subunits and (iii) the structure of the MIPS:NAD complex in the cofactor binding site. Letters with numbers indicate the amino acid in the given position in the protein using the one-letter amino acid code. d, Production of inositol lipids during growth in inositol-supplemented minimal medium (‘Glc’, exclusively glucose in medium; ‘Inos:Glc’, 1:1 molar abundance of inositol:glucose). Intensity values for the lipid peak at m/z 781.48, PI-DAG 30:0, measured in lipid extracts from WT and ΔBT_1526 strains grown either in minimal medium with glucose as the carbon source, or a 1:1 mix of myo-inositol:glucose, with n = 2 biological replicates. The inset shows higher y-axis resolution for lipid peaks from the ΔBT_1526 strain.

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terized *Renobacterium salmoninarum* PIP-DAG synthase (PIPS)\(^{23}\) revealed the same preserved catalytic residues (DX,DGX,AR… GX,DX,D) in BT_1523. This observation supports that BT_1523 functions as a PIPS in the biosynthesis of PIP-DAG.

PIP-DAG has not been reported in BT probably because PIP-DAG extractions under non-acidic conditions are low-yielding. PIP-DAG abundance may also be low in BT, as are phosphoinositides in eukaryotes.\(^1\) Following a PIP-DAG-optimized lipid extraction, we detected high levels of PIP-DAG in iSPT\(_{\Delta BT}\), which are detectable by both TLC and HPLC–MS (Fig. 2b,c, and Extended Data Figs. 1 and 2). PIP-DAG accumulation in iSPT\(_{\Delta BT}\) suggests that BT_1525 is most probably a phosphatidylinositol phosphate phosphatase (PIPPh), responsible for the rapid downstream conversion of PIP-DAG to PI-DAG. Although BT_1525 has homology to the phosphatidylycerophosphatase A protein family (Pfam), this sequence similarity could reflect an expansion of the functional role of this protein motif from the dephosphorylation of a phospholipid glycerophosphate headgroup to an inositol phosphate headgroup. Previous work has shown that transcriptomic expression of BT_1525 is higher than those of BT_1523 and BT_1522 in every growth phase of BT\(^{-}\), probably enabling the rapid conversion of PIP-DAG to PI-DAG and preventing accumulation of PIP-DAG in BT.

Despite the central location of BT_1524 in the inositol lipid metabolism gene cluster, inositol lipids in iSPT\(_{\Delta BT}\) phenocopied BT by TLC (Extended Data Fig. 3). The BT_1524 gene is predicted to encode an integral membrane protein with a Gtr-A motif (Pfam); other Gtr-A family proteins are involved in cell surface polysaccharide or exopolysaccharide synthesis.\(^{24,25}\)

To confirm that the loss of inositol lipids in knockout strains was not due to off-target effects, we genomically integrated the native BT sequence of each gene into the corresponding knockout strains in the iSPT background (iSPT\(_{\Delta BT}\), iSPT\(_{\Delta BT}\), iSPT\(_{\Delta BT}\), iSPT\(_{\Delta BT}\), iSPT\(_{\Delta BT}\), paired with a constitutive promoter optimized for BT.\(^{26}\) The complementation was successful for three of the four strains (iSPT\(_{\Delta BT}\), iSPT\(_{\Delta BT}\), and iSPT\(_{\Delta BT}\)), fully restoring the capacity for both PI-DAG and PI-DHC synthesis (Fig. 2b).

**Structural characterization of BT_1526 MIPS.** To confirm the predicted function of BT_1526 as a redox-neutral, nicotinamide adenine dinucleotide (NAD\(^+\)/NADH)-dependent MIPS, we cloned the gene and heterologously overexpressed the protein in *Escherichia coli* (Fig. 3 and Supplementary Fig. 3). The MIPS activity of BT_1526 was confirmed using a colorimetric endpoint assay (Fig. 3a,b and Supplementary Information) and the structure resolved to 2.0 Å resolution (Fig. 3c). Outside of the highly conserved ligand binding site, the overall fold and quaternary structure of the representatives of the family in the Protein Data Bank (PDB) is highly conserved, although eukaryotic MIPS proteins have an N-terminal extension that appears to further stabilize the quaternary structure of the protein (Extended Data Fig. 4 and Supplementary Fig. 4). Overall, the enzyme described here as the first MIPS representative in the Bacteroidetes retains the key functional elements of other MIPS enzymes (see Supplementary Information), underscoring its conservation across biological kingdoms.

**Inositol links to the capsule.** Given excess exogenous inositol, BT is unable to take up more than a very minor amount for inositol lipid synthesis (Fig. 3d). To characterize the roles of MIPS and inositol in BT, we analysed the iSPT\(_{\Delta BT}\) transcriptome at early stationary phase. Twenty-nine genes were differentially expressed between iSPT\(_{\Delta BT}\) and iSPT. Unexpectedly, the majority were involved in capsule biosynthesis (Supplementary Table 3). Indeed, while SL induction level had little effect on expression of capsular polysaccharide synthesis (CPS) loci in iSPT, iSPT\(_{\Delta BT}\) had notable upregulation of CPS1 and CPS6 (Fig. 4a). iSPT\(_{\Delta BT}\) is deficient in inositol lipids (PIP-DAG/PI-DAG/PI-DHC) and any other possible inositol-containing molecules, such as cell surface polysaccharides and components of the capsule. Interestingly, scanning electron microscopy (SEM) revealed heterogeneous capsule structures in iSPT and iSPT\(_{\Delta BT}\) strains, with more cells of the iSPT\(_{\Delta BT}\) strain exhibiting a dense exopolysaccharide-like structure connecting adjacent cells (Extended Data Fig. 5).

Transcriptional responses in ΔBT_1522 (lacking PI-DHC) showed no upregulation of CPS1 and CPS6, suggesting that the effect of MIPS deficiency alone caused the capsule effects in the iSPT\(_{\Delta BT}\) strain. Compared with WT, ΔBT_1522 differentially expressed 37 genes unrelated to the capsule (Supplementary Table 4), indicating that CPS regulation is related to levels of inositol phosphate, but not inositol SLs. These genes encode many hypothetical proteins and membrane-associated proteins, including those involved in carbohydrate metabolism, such as SusD starch-binding protein homologues (BT_3025 and BT_2806). Pathway enrichment analysis revealed an enrichment in ΔBT_1522 of transcripts involved in sugar degradation (specifically, 5-dehydro-4-deoxy-d-glucurionate degradation, \(P=0.006\), Fisher exact test, Benjamini-Hochberg correction), acetate and ATP formation from acetyl-CoA (\(P=0.098\)), and carboxylate degradation (\(P=0.098\)).

![Fig. 4 | Inositol synthesis influences CPS loci expression and inositol lipids alter AMP resistance and in vivo fitness. a Gene expression data (normalized log, expression values, scaled by row) in the 8 BT CPS loci. Genes were filtered to include those in which maximum log2-normalized expression is >1.5 and exclude those with maximum absolute log2-fold-change difference in expression <1.5 in all pairwise comparisons of conditions. The tree above the expression data represents the Euclidean cluster defining the sample order by expression similarity. Colour in the far right column indicates gene assignment to one of 8 CPS loci. Strains tested include WT BT, iSPT, ΔBT_1522 and ΔBT_1523. X-axis shows the time of induction (in minutes) of aTC induction is indicated in shades of grey. Labels below each column indicate strain, induction level and replicate ID according to the following pattern: ‘(strain) - (aTC induction in ng ml\(^{-1}\) - mean of 3 technical replicates) and in the caeca of 8 gnotobiotic mice following a 14 d colonization (\(n=8\) mice; each point is the mean of 3 technical replicates per mouse). The black line indicates the median of the WT BT abundance in mouse caeca following the competition experiment (73.0%).](https://www.nature.com/naturemicrobiology)
Inositol has not been previously reported as a component of BT capsule\textsuperscript{29}, perhaps due to its common use as an internal standard in the high-performance anion-exchange chromatography with pulsed amperometric detection analysis of capsule components. Using an alternative standard in gas chromatography–mass spectrometry (GC–MS), we detected very low or no inositol in $\Delta$BT\_1526's capsule (that is, 0.01\% molar abundance in one replicate of $\Delta$BT\_1526; Fig. 4b), yet detected minor amounts (at most 0.2\% molar abundance) in the capsular glycosyl residues of WT, $\Delta$BT\_1522, $\Delta$BT\_1523, $\Delta$BT\_1524 and $\Delta$BT\_1525 strains. Apart from inositol, the composition of 10 other glycosyl residues in the capsule extracted from each knockout strain (for example, glucose, rhamnose, mannose and so on) phenocopied WT (Extended Data Fig. 6). For the $\Delta$BT\_1526 strain, we observed a transcriptomic shift in CPS loci expressed to CPS1 and CPS6 (Fig. 4a), which may be expected to alter the glycosyl residue composition of the capsule\textsuperscript{29}; however, this was not observed (Extended Data Fig. 6).
Inositol lipid deficiency affects bacterial fitness. To assess the role of inositol and inositol lipids on bacterial physiology, we compared growth of each BT knockout strain. In rich medium, the different knockout strains had similar growth characteristics. In minimal medium with glucose as the sole carbon source, ΔBT_1526, in which MIP cannot be synthesized de novo, had lower cell density at stationary phase (Extended Data Fig. 7).

Alterations to the capsule and cell membrane can be expected to change bacterial susceptibility to antimicrobial peptides (AMPs) that target these structures. To test whether the presence of inositol and inositol lipids altered AMP resistance, we treated each strain with human cathelicidin LL-37, a cationic peptide expressed in the colon that is electrostatically attracted to negative membrane charges (for example, from inositol lipid and phosphatidylserine headgroups)⁴⁶. Consistent with a change in membrane charge, ABT_1523 and ΔBT_1526 had a higher half maximal inhibitory concentration (IC₅₀) for LL-37 than WT (Fig. 4d). However, ΔBT_1525 was most resistant to LL-37, possibly due to unanticipated effects of PIP-DAG accumulation.

To determine whether inositol and/or inositol lipids are important for fitness in a mammalian host, we mono-associated germ-free (GF) mice with WT, ΔBT_1522, ΔBT_1523 and ΔBT_1526. Each strain was administered to eight 4–6-week-old female C57BL/6 GF mice fed a standard chow diet ad libitum for 14 d. Previous studies have shown that BT reaches peak colonization levels of 10⁶–10⁸ cells per ml in the caecum after a 10 d colonization⁴¹, therefore we measured caecal cell density following 14 d as a measure of bacterial fitness. We observed a reduction in fitness for ΔBT_1526 compared with WT (Fig. 4d). To test for relative fitness, we competed WT and ΔBT_1523. We chose ΔBT_1523 to specifically test fitness effects of inositol lipids in vivo. GF mice were inoculated with 1:3 WT:ΔBT_1523, but despite its greater proportion in the inoculum, ΔBT_1523 had a clear fitness defect after 14 d, with an average abundance of 76% of the WT strain (n = 8, median 73%, range 62–98%; Fig. 4e).

Inositol lipid synthesis is widespread in gut Bacteroidetes. To survey for inositol lipid synthesis in the Bacteroidetes, we searched for homologues of MIPS, PIPS, PIPPh, PI-DHC synthase, and the BT InsP₆ phosphatase, MINPP₃₂ (BT_1526, BT_1523, BT_1525, BT_1522 and BT_4744, respectively) in the genomes of 10 species (Fig. 5a and Supplementary Table 5). TLC analysis of lipids from these species revealed that most had lipid bands consistent with PI-DAG and/or PI-DHC in agreement with their genomically-predicted capacity (the exception was Fluctobacillus major). However, we were surprised to also observe lipid bands consistent with the synthesis of PI-DAG and PI-DHC in species lacking homologues of BT_1522/23/25. HPLC-MS analysis of these lipids confirmed that two species genomically predicted to lack inositol lipids (B. vulgarus and P. veroralis) produced PI-DHC (Fig. 5b).

To understand why bacterial species lacking BT inositol lipid synthesis homologues nevertheless produced these lipids, we searched the genomes of related species containing a BT_1526 (MIPS) homologue but lacking the remainder of the BT cluster. Using PHI-BLAST with the conserved catalytic residues in BT_1523 (DX,DGX,AR...GX,DX,D)ⁿ, we identified a predicted CDP-alcohol phosphatidyldilferase genomically encoded near the MIPS homologue in B. vulgarus. Almost every Bacteroides/Prevotella species containing a MIPS homologue has one of two clusters directly in the vicinity of MIPS—either the BT-like cluster (BT_1522/23/25), or an alternate cluster encoding an NTP transferase (nucleotidyltransferase) domain-containing protein, CDP-alcohol-phosphatidyldilferase, and haloalkaneo dehalogenase (HAD) hydrolase (Fig. 6). The NTP transferase domain family protein (NCBI Conserved Domain Family cl11394) also shares homology with a phosphocholine cytidyldilferase motif, suggesting that this protein may synthesize cytidine 5’-diphosphoinositol (CDP-inositol), similar to the synthesis of CDP-inositol as a precursor to di-my-inositol phosphate solutes and dialkylether glycerophosphoinositol lipids in hyperthermophiles⁴³,⁴⁴. The HAD hydrolase superfAMILY is large and diverse, with the majority of characterized members functioning as phosphotransferases⁴⁵. As a lipid phosphate phosphohydrolase, this HAD hydrolase may function similarly to AUR1⁴⁶, acting as a PI-DHC synthase such as BT_1522.

The functions of the NTP transferase domain protein, CDP-alcohol-phosphatidyldilferase, and HAD hydrolase are not confirmed but offer an alternative pathway to enable synthesis of PI-DHC without a PIP-DAG intermediate (similar to PI-DAG synthesis in euarkyotes⁴⁷), with PI-DAG synthesis resembling the synthesis of phosphatidylethanolamine or phosphatidylcholine in the Kennedy pathway⁴⁸⁴⁹. Following this logic, the NTP transferase protein would first synthesize CDP-inositol from myo-inositol phosphate and CTP. CDP-inositol and a diacylglycerol (DAG) substrate would then be converted to PI-DAG by the CDP-alcohol-phosphatidyldilferase, and PI-DAG would be converted to PI-DHC by the HAD hydrolase (see pathway comparison in Extended Data Fig. 8). The MIPS homologue is most commonly clustered directly with these other genes, with some exceptions (for example, P. copri (Fig. 6). Interestingly, in P. veroralis, the CDP-alcohol phosphatidyldilferase and HAD hydrolase proteins are fused (Supplementary Fig. 5), suggesting the possibility for a cohesive single-enzyme conversion of CDP-inositol to PI-DHC through a PI-DAG intermediate. Some additional putative enzymes are fused in the vicinity of both clusters, including those annotated as a lysylphosphatidylglycerol synthase (BT_1521 homologue) and a carboxypeptidase-regulatory-like domain protein (BT_1527 homologue) (Fig. 6). This alternative pathway could explain PI-DHC synthesis by P. veroralis and B. vulgarus despite their lack of homologues to the BT inositol lipid cluster (BT_1522/23/25).

Among the Proteobacteria species tested, Sphingomonas paucimobilis and Novosphingobium acidiphilum made only non-hydroxylated SLs (Extended Data Fig. 9). Despite lacking homology to either the BT-like or putative alternative inositol lipid cluster, N. acidiphilum produced an SL with a retention time and headgroup fragmentation consistent with Bacteroides PI-DHC fragmentation (Extended Data Fig. 9a). S. paucimobilis also produced a lipid with fragmentation similar to Bacteroides PI-DHC, but lacking the fragment at 241 m/z, tentatively suggesting a phosphorylated-hexose DHC unlike those produced by Bacteroides (Extended Data Fig. 9b).
characterization of this lipid’s phosphohexose identity would, however, require purification and analysis beyond the scope of this study. In addition, the TLC analysis (Fig. 5a) shows a lipid band in the PI-DAG/PI-DHC region for mouth-associated *Porphyromonas gingivalis*, which is probably a phosphorylglycerol-DHC (Extended Data Fig. 9c).
To assess the distribution of the BT inositol lipid cluster (BT_1522/23/25) and the potential alternative pathway among the Bacteroidetes, we searched for homology in 162 representative species of Bacteroidetes (Supplementary Table 5). Most strains with a homologue of BT_1522, BT_1523 or BT_1525 have homologues of all three, but the distribution does not track phylogeny, supporting lateral exchange among host-associated species (Fig. 6). Roughly three-quarters of Bacteroides, Prevotella and Parabacteroides species have a MIPS homologue paired with either the BT-like inositol lipid cluster or the putative alternative cluster. One notable exception is...
**Discussion**

Inositol lipids have only recently been reported in a few commensal gut bacteria. In this study, we characterized the gene cluster recently hypothesized to be involved in bacterial inositol lipid synthesis in BT to show a functional role for these genes in the Bacteroidetes. BT synthesizes PI-DAG using a mycobacterial-like pathway with a PIP-DAG intermediate; previously, the bacterial PI-DAG synthesis pathway lacked a PIPPh, which we have identified here as BT_1525. We also identified a putative alternative pathway for PI-DHC synthesis, common among *Prevotella* species, that lacks a PIP-DAG intermediate, resembling the eukaryotic Kennedy pathway for phosphatidylethanolamine and phosphatidylcholine synthesis. The majority of host-associated Bacteroidetes species encode one or the other of these pathways, indicating that inositol lipid production is a fundamental trait in the phylum. Together with the importance of inositol lipids in pathogen–host interactions and their impact in this work on fitness in a mammalian host, their high prevalence in the host-associated Bacteroidetes suggests an unexplored role for inositol lipids in commensal–host interactions.

We noted a link between inositol production and the BT capsule, which contained very small amounts of inositol. Importantly, we also detected inositol in capsule from ΔBT_1523, which lacks inositol lipids, indicating that the presence of inositol is not solely lipid-linked. Despite its low capsular abundance, inositol may play an important structural role, as observed in *Mycobacteria*, where >100 glycosyl residues can be bound to a single lipid-linked inositol. However, the low inositol abundance in our capsule analysis lacks enough statistical power to determine this unequivocally in the BT. The CPS loci expressed by BT, and differentially expressed when MIPS is deficient, have been shown to influence recognition by the host adaptive immune system and bacteriophage. The strong effect of MIPS deficiency on transcription of capsule-related genes may therefore indicate an indirect role for inositol on cross-kingdom interactions.

Plants and yeasts also produce inositol-linked SLs critical for fundamental aspects of the organism’s physiology, such as protein anchoring and programmed cell death. The yeast homologue of BT_1522 is an antifungal target inhibited by the cyclic depsipeptide natural product aureobasidin (hence the name AUR1). Although ΔBT_1522 had few overall transcriptomic changes relative to WT controls, the affected pathways are central to carbohydrate degradation and energy synthesis. This result indicates that similar to these more distantly related organisms, inositol SLs appear to influence pathways central to bacterial physiology.

Our results show that deficiencies in inositol and/or inositol lipids affect interactions with AMPs secreted by the mammalian host and its fitness in vivo. Importantly, deficiency in the ability to produce inositol cannot be rescued by assimilation of inositol from the growth medium and impacts growth both in vitro and in vivo. Taken together, our results indicate that inositol and inositol lipids are probably implicated in resistance to host immune defences through their roles in the structure of the membrane and the capsule, and important for fitness in the mammalian gut.

Our comparative genomic analyses revealed inositol lipid synthesis to be far more widespread in host-associated Bacteroidetes than previously thought. Although the putative alternative pathway remains to be functionally confirmed, the vast majority of species investigated encoded one of the two pathways, with the alternative pathway being more common in *Prevotella*. The extensive prevalence of this function is in agreement with the widespread capacity in gut commensals for phytate (InsP6) degradation, which releases phosphorylated inositol derivatives. Although InsP6 phosphatase is rare across Bacteria (present in only 2.2% of completed genomes in the European Bioinformatics Institute database in 2014), the majority of these enzymes are found in gut microbiome-affiliated species. In addition to the widespread capacity for de novo synthesis of inositol and its lipids reported here, these observations suggest that inositol and inositol lipid cycling in the gut are fundamental attributes of the gut microbiome.

Of the six dominant *Bacteroides* species in the human gut, five have genes with homology to the BT-like inositol lipid cluster (*B. cellulosilyticus*, *B. eggerthii* and *B. ovatus*) or the putative alternative cluster (*B. dorei* and *B. vulgatus*), indicating potential for inositol lipid synthesis. As one of the most abundant phyla within the human gut, the widespread synthesis of inositol lipids from gut-associated Bacteroidetes (*Bacteroides*, *Prevotella* and *Parabacteroides* spp.) could represent a significant contribution to the lipid milieu of the gut. Bacterial lipids with high structural similarity to eukaryotic bioactive lipids (for example, SLs) have been shown to influence the metabolism and immune homoeostasis of their host. Likewise, bacteria are already known to manipulate their host through inositol and inositol lipid metabolic pathways, and many bacterial and viral pathogens have also adapted to hijack the host phosphoinositide system. Our work suggests that inositol and inositol lipid metabolism are prevalent in host-associated Bacteroidetes and represent an unappreciated means of cross-kingdom communication, with effects on the host that remain to be ascertained.

**Methods**

**Bacterial strains and cultivating conditions.** Unless otherwise stated, all liquid *B. thetaiotaomicron* VPI-5482 Δtak (WT BT) cultures were grown anaerobically (95% N2 and 5% CO2 atmosphere) at 37°C in supplemented BHI media (BHS;
37 g L−1 brain-heart infusion, 5 g L−1 yeast extract, 1 mg L−1 menadione, 1 mg L−1 resazurin, 10 mg L−1 hemin and 0.5 g L−1 cytochrome HCl. E. coli cultures were grown aerobically at 37°C in Luria broth with shaking. Final concentrations of antibiotics and selection agents were as follows: erythromycin 25 μg mL−1, gentamicin 200 μg mL−1, streptomycin 100 μg mL−1, carbenicillin 100 μg mL−1 and 5-fluoro-2′-deoxyuridine 200 μg mL−1. In select experiments, BT was grown in Bacteroides minimal media (BM4); per litre: 13.6 g KH2PO4, 0.875 g NaCl, 1.2 g (NH4)2SO4, 5 g NaNO3, 5 g glucose (pH to 7.2 with concentrated NaOH), 1 ml hemin (500 mg dissolved in 10 ml of 1 M NaNO3 then diluted to final volume of 500 ml with water), 1 ml MgCl2 (0.1 M in water), 1 ml FeSO4.7H2O (1 mg per 10 ml of water), 1 ml vitamin K3 (1 mg mL−1 in absolute ethanol), 1 ml CaCl2 (0.8% w/v), 250 μM vitamin B12 solution (0.02 mg mL−1) and 0.5 g L−1 cytochrome HCl.

For lipid analysis of non-BT strains: S. paucimobilis (ATCC 29837) was grown aerobically at 30°C in nutrient broth (per litre: 5.0 g peptone and 3.0 g meat extract; pH 7.0). B. fragilis (DSM 2151), Porphyromonas gingivalis (DSM 20709), B. unifors (DSM 6697), B. vulgatus H5_1 (DSM 108228), B. vulgatus (DSM 1447), Prevotella veroralis (ATCC 35779), Phociccola dorei (DSM 17855), Prevotella nigrescens (DSM 19666) was grown at 37°C in DSM medium 104 at 37°C. F. major (DSM 103) was grown at 26°C in DSM medium 7 (per litre: 1.0 g glucose, 1.0 g peptone and 1.0 g yeast extract; pH 7.0). Acetobacter malorum (DSM 14337) was grown at 28°C in DSM medium 360 (per litre: 5.0 g yeast extract, 3.0 g peptone and 25.0 g mannitol). N. acidipilum (DSM 19666) was grown at 28°C in DSM medium 1199 (per litre: 1.0 g glucose, 1.0 g yeast extract and 1.0 g peptone; pH 5.5).

Generation of BT knockouts and inducible SPT strain. Genetic manipulations in the B. thetaiotaomicron VPI-5482 ΔtetK (WT) strain were performed using double recombinant from a suicide plasmid as previously described. The generation of the BT_0870 (SPT) knockout has been previously described. To create the inducible SPT (ispt) strain, three TetR cassettes were inserted into the ΔtetK_0870 genome with the constitutive PBT1311 promoter as previously described, with the native SPT (B. thetaiotaomicron VPI-5482 ΔtetK_0870) sequence reintroduced under the inducible PTTDP promoter. Induction of SPT was performed using anhydrotetracycline (aTC) (Cayman 19556) and d18:0 sphinganine (Avanti 860498).

LC/MS analysis of PIP-DAG lipids for acyl chain determination. To identify the acyl chain distribution in PIP-DAG lipids, WT and ΔtetK_1526 strains were grown and lipid extracted (using the PIP-DAG-specific acidic extraction) as described above. A Dionex UltiMate 3000 HPLC system (ThermoFisher) coupled with a high-resolution mass spectrometer with an electrospray ionization source (Impact II mass spectrometer, Bruker) was used for the analysis of the lipid extracts. The separations were performed on a hydrophilic interaction liquid chromatography column (LC inner diameter 150 mm x 5 mm, 2.6 μm particle size) held at a constant temperature of 60°C. The mobile phase consisted of solvent A (0.1% formic acid and 2 mM ammonium formate in water:acetonitrile 40:60 (v/v)) and solvent B (0.1% formic acid and 2 mM ammonium formate in methanol). The following gradient: A/B 35/65 (0 min), 35/65 (from 2nd min), 60/40 (from 4th min), 60/40 (from 6th min), 30/70 (from 8th min) and 25/75 (at 10th min) was applied for elution with a constant flow rate of 0.6 mL min−1. The injection volume was 1 μL. The operating parameters of the mass spectrometer were as follows: the spray needle voltage was set at 3.5 kV, nitrogen was used both as the nebulizing gas (1.5 bar) and the drying gas (5 min−1), and the drying temperature was 200°C. Data were acquired in data-dependent acquisition mode, with the 3 most intense parent ions chosen for MS/MS acquisition. The scanning range was 50–1,500 m/z and the scanning rate was 2 Hz in the negative ion mode. The collision energy was 65 eV, with nitrogen used as collision gas. Full MS chromatogram was used for the quantification purpose (Skyline software version 21.1), while the MS/MS spectrum was used for identification and/or confirmation of PIP-glycerolipid (Compass Data Analysis version 4.3).

Fatty acid methyl ester (FAME) analysis of lipid acyl chains by GC–MS.

Bacterial cultures were inoculated from overnight stationary cultures 1:500 (v/v) into 100 ml BHIS media and grown for 16 h at 37°C, to an OD600 of 0.30–0.45. Fatty acid methyl esters (FAME) were prepared by reaction with methanol:chloroform for 10 min. The resulting pellets were extracted into 2.25 ml methanol:chloroform:12 N HCl (80:40:1) and 0.75 ml chloroform, combined with 1.35 ml 0.1 N HCl and vortexed. The lower fraction was dried under nitrogen and resuspended in 20:9:1 chloroform:methanol:water for TLC.

The lipid extracts were applied to a silica HPTLC plate with concentration zone (Supelco 60768), with loading volumes normalized to the optical density (OD)max of original cultures. Plates were developed in a 62:25:4 (v/v/v) chloroform:methanol:ammonium hydroxide (23% NH3 basis) system (for standard lipid extractions) or 48:40:7.5 chloroform:methanol:water:ammonium hydroxide (for PIP-DAG extractions), then sprayed with primuline (0.1 mg mL−1 in 4:1 v/v acetonitrile:distilled H2O), and imaged under ultraviolet transillumination (365 nm). TLC densitometry of scanned plates was used to measure the proportion of SLs (Supelco 60768), with loading volumes normalized to the concentration zone (Supelco 60768), with loading volumes normalized to the concentration.
and 800 μl hexane were added, followed by vortexing. After centrifugation for 5 min at 4,000 × g, the upper layer was removed under a Mosquito robot (SPT Labtech) using the sitting drop vapour diffusion method. The plates were incubated at 20 °C and the initial hits were suitable for differentiation experiments. The condition yielding crystals that were subjected to X-ray diffraction was PACT F6 (Molecular Dimensions, 200 μm sodium formate 100 mM bis tris propane pH 6.5 and 20% (v/v) PEG 3350). The sample was cryoprotected with the addition of 20% PEG 400 to the reservoir solution.

Data collection, structure solution, model building, refinement and validation of BT_1526. Diffraction data were collected at the synchrotron beamline I04 of Diamond light source at a temperature of 100 K. The data set was integrated with XDS and indexed with DIAlS-2 and scaled with Aimless. The space group was determined with Pointless and manual model building was done with COOT. The model was refined with refmac and manual model building was done with COOT. All other software used were from CCP4 and the CCP4 suite.

Inositol uptake. To determine whether BT is capable of taking up exogenous inositol and incorporating this into lipids in the absence of MIPS/BT_1526, we prepared BMM containing solely glucose as a carbon source or an equivalent molar mass of 1.1 myo-inositol:glucose. WT and ΔBT_1526 strains were inoculated into each medium from dense overnight cultures (n = 2) and grown for 16 h at 37 °C. Lipids were extracted using the Folch method (standard (non-acidic) lipid extraction, described above) and lipids were measured by MALDI MS.

For MALDI MS analysis, lipids (100 μg ml⁻¹ in MeOH) were mixed: 1:1 (v/v) with 1.5-diaminonaphthalene (10 mg ml⁻¹ in acetone/water/trifluoroacetic acid 60:39:1, v/v) and spotted onto the MTP 384 ground steel target and allowed to dry at room temperature. Measurements were performed using a Bruker ScimaX 7T 2xR FTICR mass spectrometer and fimsControl V2.3.0 (Bruker Daltonics). Before the measurement, external quadratic calibration was performed with sodium formate (5 mM in 2-propanol/water 90:10 (v/v)) injected at a flow rate of 120 μl h⁻¹ (nebulizer set to 1.5 bars with a dry gas flow and temperature of 41 °C and 200 °C, respectively) using the ESI source.

All data were acquired in negative ion mode using the MALDI ionization source. Laser intensity was set to 12%. Per spot, 50 laser shots and a frequency of 104 Hz was used. Spectra were acquired with small laser focus and a smart walk of 0.10 mm from 9 to 9 offset set to 0.50 mm and expressed in the m/z range of 110–2000, with Q1 set to m/z 800. For ion transfer, voltages of funnel and Skinner 1 set to −150 V and −15 V, respectively, with the funnel radio frequency amplitude adjusted to 70 Vpp. Octopole frequency and radio frequency amplitude were set to 5 MHz and 350 Vpp, respectively. The frequency of the transfer optics was set to 4 MHz and a time of flight of 1 ms was used. Mass accuracy was ensured using the prominent lipid peak at m/z 66.476082 (PE300− H₂); C₂₃H₂₄O₅P as a reference mass for single-point calibration. The resulting spectra were processed with Bruker Compass DataAnalysis V5.2 software (Bruker Daltonik). Average intensities for 8 spots were calculated for each sample.

Growth curve analysis and LL-37 resistance. All growth curves were performed in 96-well format in an anaerobic chamber (95% N₂, 5% CO₂ atmosphere). Plates were incubated at 37 °C and OD₅₆₀ measured every 8 min for 24 h in a Victor Nivo plate reader (PerkinElmer). For growth curves: to determine alterations to growth dynamics in the knockout strains of the inositol lipid cluster, dense overnight cultures of each BT strain in BHIS were centrifuged at 16,000 × g for 1 min, the supernatant removed and the cells resuspended in an OD-normalized volume of BHIS or BMM before aliquotting 200 μl per well in a sterile 96-well plate (n = 4). For AMP resistance: to identify changes in each strain’s sensitivity to the AMP LL-37, dense overnight cultures of each BT strain were OD-normalized and diluted to a starting OD₅₆₀ of ~0.15 in a total of 200 μl BMM per well, each well supplemented with 0–64 μg ml⁻¹ LL-37 (InViVoGen) in duplicate per strain and AMP concentration. Inhibition due to LL-37 was graphically modelled by percent OD at 0 ng ml⁻¹ LL-37: (maximum OD in 24 h − minimum OD in 24 h)/ average(maximum − minimum OD at 0 ng ml⁻¹ per strain) × 100. IC₅₀ was calculated in Prism by fitting concentration inhibitor vs normalized response with variable slope and between-strain comparisons using Tukey’s multiple comparisons. The data shown are representative of two experiments.

RNA-seq of BT at varied levels of SPT induction. Overnight cultures were used to inoculate (in duplicate) BMM media 1:2,500 (v/v) uninoculated or at 1 of 5 varied SPT concentrations (0, 0.2, 0.5, 1.0, 5.0, 100 ng ml⁻¹), and incubated at 37 °C for 15 h to an OD₅₆₀ of 0.10–0.17. Cultures were spun at 3,500 × g for 15 min and RNA was extracted from the bacterial pellet with QIAzol lysis reagent and the miRNAeasy mini kit (Qiagen). Ribosomal RNA was removed with the Bacterial RiboMinus transcription isolation kit (Invitrogen) and the library prepared with the TrueSeq stranded total RNA library kit (Illumina); libraries were pooled at 9 per lane and sequenced by HiSeq3000 (Illumina).

Quality assessment of reads was performed using FastQC pre- and post-quality filtering with bbduk (quality cut-off 20). Reads were aligned to the Ensembl B.
Phylogenies of homolog to BT inositol lipid metabolic enzymes in diverse bacteria. For the smaller phylogeny of diverse sphingolipid producers (34), homology to BT inositol and inositol lipid metabolism enzymes BT_1522, BT_1523, BT_1525, and BT_1526 in the indicated species was identified using NCBI BlastP83. For the larger phylogeny of Bacteroidetes and related genera (Fig. 6), all representative species for Bacteroides, Prevotella, Parabacteroides, Porphyromonas, Flavobacterium, Sphingobacterium and Flavobacterium genera with nomenclature recognized in the List of Prokaryotic names with Standing in Nomenclature (LPSN)83 were tested for homology to BT inositol metabolism enzymes BT_1522, BT_1523, BT_1525, BT_1526 and BT_4744. For phylogenetic comparison in both trees, 71 single-copy genes present in all genomes (HMM profile Bacteria, 71) were identified and concatenated using Anvi’o85, with alignment using MUSCLE85. RAxML86 was used to generate a maximum-likelihood tree (Protcat substitution model, Dayhoff matrix, Hill-climbing algorithm, 50 bootstrap iterations). Strain accession numbers and BlastP results are in Supplementary Table 5.

Statistics and reproducibility. Statistical significance for cellular colonization and competition experiments, antimicrobial peptide (LL-37) resistance and capsule composition was calculated by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons. Statistical analysis was performed with GraphPad Prism version 9.0 and statistical significance is indicated by: **P < 0.01; ***P < 0.001; **P < 0.01; **P < 0.05; NS (not significant) P > 0.05. In the RNA-seq experiment, P values were calculated from trimmed mean of M values TMM and adjusted according to the Benjamini-Hochberg method; only significantly differentially expressed genes with >1.5 absolute log fold change are reported. The 'n' reported in each experiment represents an individual biological replicate for the relevant experiment (for example, mouse caecal sample, bacterial culture). No statistical methods were used to pre-determine sample sizes, but our sample sizes are similar to those reported in previous publications87. Data collection and analysis were not performed blind to the conditions of the experiments. No animals or data were excluded from the analyses. In mouse experiments, mice were randomly assigned to a treatment condition to randomize age and litter of origin. For in vitro experiments, following strain generation, identical treatments were performed (for example, lipid extraction and analysis, capsule extraction and analysis, AMP resistance and growth curves) so randomized allocation was not necessary. TEC experiments were repeated independently at least two times with similar results.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The BT_1526 MPs structure analysed during the current study is available in the Protein Data Bank repository, PDB ID 7NRW. RNA-seq data and reads are available at NCBI GEO (https://www.ncbi.nlm.nih.gov/geo) under accession number GSE193734. Mass spectrometry files and all unique strains generated in this study are available from the corresponding author upon request. All remaining data generated during this study and its Supplementary Information. Source data are provided with this paper.

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Author contributions
S.L.H. and R.E.L. conceived and planned the experiments. P.T. purified MIPS and performed its kinetic assay with input from D.J.C. J.M.-W. and A.B. performed the structural studies of MIPS. H.H.L., C.M.B. and D.L.V. performed mass spectrometry with input from E.L.J. J.L.W. coordinated the mouse experiment. S.L.H. performed all other experiments (strain generation, bacterial culturing, lipid extractions, TLC, FAME analysis, RNA-seq, capsule extractions, AMP resistance assays, mouse analyses and bioinformatic/phylogenetic analyses), analysed the data and wrote the first draft of the manuscript. S.L.H. and R.E.L. prepared the final manuscript. All authors provided comments and gave approval for publication.

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Lipid structures and fragmentation patterns of BT inositol and ethanolamine lipids. (A) Comparison of LC-MS/MS fragmentation patterns of ΔBT-IS25 BT-derived phosphatidylinositol phosphate (PIP-DAG) with the synthetic standard, PI(3)P 18:1/18:1. (B) LC-MS/MS fragmentation patterns of lipid structures present in iSPT BT at 100 ng/mL aTC induction, including phosphoinositol dihydroceramide (PI-DHC) lipids (PI-DHC 34:0(OH), PI-DHC 35:0(OH), PI-DHC 36:0(OH)), PI-DAG 30:0, and PE-DHC 36:0(OH). Loss of the phosphoinositol head group is indicated at mass 259. Fragments characteristic for lipids with phosphoinositol-based headgroups are in red; those for phosphoethanolamine-based headgroups are in blue. Branching patterns of DHC-based lipids are predicted.
Extended Data Fig. 2 | Summary of lipid species and inositol metabolites produced by each WT or knockout strain. Lipid color scheme is consistent with Fig. 2. Red x = not present; green check = present. Inositol-P ("inos-P") is described as present/absent dependent on the presence of the BT_1526 gene. PIP-DAG = phosphatidylinositol phosphate; PI-DAG = phosphatidylinositol; PI-DHC = phosphoinositol dihydroceramide; PE-DHC = phosphoethanolamine dihydroceramide; PE-DAG = phosphatidylethanolamine. Yellow background for PIP-DAG presence/absence indicates PIP-DAG presence (determined by presence of downstream metabolites), but these lipids are not detectable by mass spectrometry, due to predicted fast turnover.
**Extended Data Fig. 3** Lipid comparison of the ΔBT_1524 strain compared to iSPT. TLC of lipid standards and Folch (non-acidic) lipid extractions from iSPT and ΔBT_1524 strains of BT. Lanes 1–8, left to right: LPC = 16:0 lysophosphatidylcholine; PI-DAG = 16:0 phosphatidylinositol; PC = 16:0 phosphatidylcholine; CPE = ceramide phosphoethanolamine; PE = egg yolk phosphatidylethanolamine; CL = cardiolipin (from bovine heart); Sa = d18:0 sphinganine; Cer = d18:1/18:0 ceramide. Following the dashed white line, in lanes 9–10, are iSPT BT lipid extracts from cells grown with 0 or 100 ng/mL aTC induction of SPT. In the final two lanes (11–12) are lipid extracts from ΔBT_1524 BT. “A” and “B” refer to independently generated knockout strains, both confirmed by Sanger sequencing. The retention factors for inositol lipids (phosphatidylinositol “PI-DAG” and phosphoinositol dihydroceramide “PI-DHC”) are indicated by labels to the right of the figure.
Extended Data Fig. 4 | Structural comparison of prokaryotic and eukaryotic MIPS proteins. Secondary structure alignment of MIPS BT_1526 (in yellow) and *Saccharomyces cerevisiae* MIPS (PDBID: 1P1I) (in cyan). The N-terminal extension present in eukaryotic MIPS structures is highlighted with a gray oval.
Extended Data Fig. 5 | Scanning electron microscopy of the iSPT and ΔBT_1526 strains. Cells were grown in minimal medium at 0 and 100 ng/mL anhydrotetracycline (aTC) induction of SPT prior to imaging by the Max Planck for Biology Tübingen Electron Microscopy Core Facility. Images are representative of multiple images derived from a single experiment.
Extended Data Fig. 6 | Capsular components of WT and inositol lipid knockout strains. (A-D) Glycosyl residues detected in the capsular extraction from each strain, presented as percent molar abundance of total glycosyl residues detected (n = 3 biological replicates per strain tested; data represented as mean values ± S.D.), in separate figures to better compare relative abundances between residues at similar concentrations. Values are in Source Data Table 6. Glc = glucose; GalNAc = N-acetylgalactosamine; GlcNAc = N-acetylglucosamine; Rha = rhamnose; GlcA = glucuronic acid; GalA = galacturonic acid; Man = mannose; Gal = galactose; Fuc = fucose; Xyl = xylose; Inos = inositol. (E) Fatty acid species detected in the capsular extraction from each strain, presented as percent abundance of total fatty acids detected (n = 3 biological replicates per strain tested; data represented as mean values ± S.D.). “15:0(OH)” is in quotations due to uncertainty of its identity - this lipid had a diagnostic signal for a 3-OH fatty acid eluting at RT = 34.800 min, however we could not detect a diagnostic m/z EI fragment (329) characteristic for hydroxypentadecanoic acid (15:0(3:OH)), instead finding an EI fragment at m/z 319.
Extended Data Fig. 7 | Growth curves of inositol lipid knockout strains in rich and minimal media. (A) Growth curves of WT BT and inositol lipid knockout strains in rich medium (BHIS) at 37 °C in anaerobic conditions (all curves with average of n = 3 shown), measured in 96-well format as optical density at 600 nm (OD600). (B) Strain growth in minimal medium (BMM), inoculated using cultures previously growing in rich medium (BHIS).
Extended Data Fig. 8 | Pathway comparison between the BT inositol lipid cluster and the predicted alternative inositol lipid pathway. At left, the BT-like inositol lipid synthesis pathway defined in this work. At right, the putative inositol lipid synthesis pathway genomically predicted for multiple Bacteroidetes spp., with predicted enzymes (in purple) from the Bacteroides vulgatus genome (NCBI reference sequence NC_009614.1). Inositol glycerophospholipids are on a gray background with green text; inositol sphingolipids are on a white background with red text. Branching patterns of BT DHC-based lipids and B. vulgatus lipids are predicted and not confirmed. SPT = serine palmitoyltransferase; MIPS = myo-inositol phosphate synthase; PIPPh = phosphatidylinositol phosphate phosphatase.
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | Non-hydroxylated inositol-like lipid structures in diverse sphingolipid-producing species. (A) LC-MS/MS fragmentation patterns of lipids extracted from *Novosphingobium acidiphilum* consistent with the synthesis of PI-DHC 35:0 and 36:0. (B) LC-MS/MS fragmentation pattern of lipids extracted from *Sphingomonas paucimobilis*, demonstrating the presence of a headgroup with the same mass as inositol phosphate (259) but lacking the characteristic fragment of this group (241). As such, the headgroup identity remains tentative and is represented with a phosphohexose. (C) LC-MS/MS spectra and fragmentation pattern of a P(Glycerol)-DHC 36:0 structure present in *Prevotella copri*, *Porphyromonas gingivalis*, and *Bacteroides vulgatus*. Branching patterns and acyl chain distribution of lipid structures shown are possible representative structures and are not confirmed.
## Reporting Summary

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For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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| For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted Give P values as exact values whenever suitable. |           |
| For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings |           |
| For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes |           |
| Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated |           |

Our web collection on statistics for biologists contains articles on many of the points above.

### Software and code

Policy information about availability of computer code

| Data collection | For mass spectrometry, Skyline version 21.1 and Compass Data Analysis version 4.3 were used. NCBI Blast+ V2.11.0 was used for homology searches. |
|-----------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data analysis   | Mass spectrometry: Spectra were processed with MassLynx (V4.1). RNA-seq: Quality assessment of reads was performed using FastQC (V0.11.8) pre- and post-quality filtering with bbduk (V38.90). Reads were aligned with bowtie2 (V2.3.5.1) and assigned using htsq-count (V0.11.2). Differential expression analysis was performed with EdgeR (V3.32.1) and limma (V3.46.0). Heatmaps were generated with heatmap (V1.0.12). Crystallography: The data set was integrated with XIA2 (V0.3.7.0) using DIALS (V3.9.1) and scaled with Aimless (V0.3.6). The space group was confirmed with Pointless (V1.10.20). The phase problem was solved by molecular replacement with Phaser (V2.7.17). The model was refined with reffmac (V5.8) and manual model building with COOT (V0.1.2). The model was validated using COOT and Molprobity (V4.5). Figures were made with ChimeraX (V1.2). |

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
Data

Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
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The BT_1526 MIPS structure analyzed during the current study is available in the Protein Data Bank repository, PDB ID:7NWR. RNA-seq reads and data are available at NCBI GEO (https://www.ncbi.nlm.nih.gov/geo/) under accession number GSE193734. Mass spectrometry files, and all unique strains generated in this study are available from the corresponding author upon request. All remaining data generated during this study are included in this published article and its supplementary information files.

Field-specific reporting

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Life sciences

Sample size
The mouse experiment used n=8 mice per strain or competition pair tested, to minimize the number of mice used in a pilot test. We initially planned to use n=12 mice per group, in alignment with the mouse group size our lab has previously used for statistical testing of bacterial lipid-dependent physiological effects (1). We obtained however ethical approval for n=8 mice per group, as this was a pilot experiment, and this group size was sufficient to make our final conclusions. Capsule analysis was performed with n=3 capsule extractions per strain to compromise between cost of analysis and low overall abundance of the target of interest. On the basis of past studies showing significant results with the given sample size (2-4), RNA-seq analysis was performed with two biological replicates per strain, which we deemed to be sufficient due to low variability between replicates.

1. Johnson, E. L. et al. Sphingolipids produced by gut bacteria enter host metabolic pathways impacting ceramide levels. Nat. Commun. 11, 2471 (2020)
2. McNulty, N. P. et al. Effects of diet on resource utilization by a model human gut microbiota containing Bacteroides cellulosilyticus WH2, a symbiont with an extensive glycosome. PLoS Biol. 11, e1001637 (2013)
3. Kijner, S., Cher, A. & Yassour, M. The Infant Gut Commensal Bacteroides dorei Presents a Generalized Transcriptional Response to Various Human Milk Oligosaccharides. Front. Cell. Infect. Microbiol. 12, 854122 (2022)
4. Dodd, D., Moon, Y.-H., Swaminathan, K., Mackie, R. I. & Cann, I. K. O. Transcriptomic Analyses of Xylan Degradation by Prevotella bryantii and Insights into Energy Acquisition by Xylanolytic Bacteroidetes. J. Biol. Chem. 285, 30261–30273 (2010)

Data exclusions
No data were excluded from the analyses.

Replication
With the exception of the mouse colonization, MIPS crystallization, electron microscopy, and RNA-seq, each experiment was performed a minimum of two times. Using the mouse samples, cecal colonization and in mouse-competition analyses were also performed twice. All attempts at replication were successful and supported the conclusions in the manuscript.

Randomization
For in vitro experiments, following strain generation, identical treatments were performed (e.g., lipid extraction and analysis, capsule extraction and analysis, AMP resistance and growth curves) so randomized allocation was not necessary, as a physical value was measured which is not influenced by the observer. Growth phase, temperature, and medium for in vitro bacterial growth were controlled and all conditions of a given experiment were performed in parallel. Electron microscopy images chosen are representative of the cell population of that strain. For mouse experiments, mice were randomly assigned to a treatment condition to randomize age and litter of origin.

Blinding
The investigators were not blinded during data collection. In the mouse experiment, the same investigators inoculated the mice and collected and analyzed downstream samples, preventing blinding. During computational analysis (RNA-seq), all data were subjected to the same analysis pipelines regardless of condition. During in vitro work with the strains, blinding was not used as the work was performed by a single investigator.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.
Materials & experimental systems

- Antibodies
- Eukaryotic cell lines
- Palaeontology and archaeology
- Animals and other organisms
- Human research participants
- Clinical data
- Dual use research of concern

Methods

- ChIP-seq
- Flow cytometry
- MRI-based neuroimaging

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | Female germ-free C57BL/6 mice aged 4-6 weeks, housed with a 12 hour light:dark cycle at 22±2 deg. C and ~55±10% humidity |
|--------------------|------------------------------------------------------------------------------------------------------------------|
| Wild animals       | The study did not involve wild animals.                                                                          |
| Field-collected samples | The study did not utilize field-collected samples.                                                                  |
| Ethics oversight   | The mouse experiment was performed in accordance with the German legislation on protection of animals with permission to conduct the study obtained from the regional animal welfare committee of the Eberhard Karls Universität Tübingen, registration number EB 03/21 M. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.