Production of α-Galactosylceramide by a Prominent Member of the Human Gut Microbiota

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Abstract

While the human gut microbiota are suspected to produce diffusible small molecules that modulate host signaling pathways, few of these molecules have been identified. Species of Bacteroides and their relatives, which often comprise >50% of the gut community, are unusual among bacteria in that their membrane is rich in sphingolipids, a class of signaling molecules that play a key role in inducing apoptosis and modulating the host immune response. Although known for more than three decades, the full repertoire of Bacteroides sphingolipids has not been defined. Here, we use a combination of genetics and chemistry to identify the sphingolipids produced by Bacteroides fragilis NCTC 9343. We constructed a deletion mutant of BF2461, a putative serine palmitoyltransferase whose yeast homolog catalyzes the committed step in sphingolipid biosynthesis. We show that the Δ2461 mutant is sphingolipid deficient, enabling us to purify and solve the structures of three alkaline-stable lipids present in the wild-type strain but absent from the mutant. The first compound was the known sphingolipid ceramide phosphorylethanolamine, and the second was its corresponding dihydroceramide base. Unexpectedly, the third compound was the glycosphingolipid α-galactosylceramide (α-GalCer), which is structurally related to a sponge-derived sphingolipid (α-GalCer, KRN7000) that is the prototypical agonist of CD1d-restricted natural killer T (iNK) cells. We demonstrate that α-GalCer has similar immunological properties to KRN7000: it binds to CD1d and activates both mouse and human iNK cells both in vitro and in vivo. Thus, our study reveals BF2461 as the first known member of the Bacteroides sphingolipid pathway, and it indicates that the committed steps of the Bacteroides and eukaryotic sphingolipid pathways are identical. Moreover, our data suggest that some Bacteroides sphingolipids might influence host immune homeostasis.

Introduction

Sphingolipids and their breakdown products modulate a variety of eukaryotic signaling pathways involved in proliferation, apoptosis, differentiation, and migration (Figure 1). Although sphingolipids are ubiquitous among eukaryotes, few bacteria produce them [1]. The genus Bacteroides and its relatives are an important exception; 40%–70% of the membrane phospholipids of these prominent symbionts are sphingolipids [2,3]. While the structures of several Bacteroides sphingolipids have been solved, the full repertoire of these molecules has not yet been defined [1–19]. Here, by systematically exploring the sphingolipid repertoire of Bacteroides fragilis, we show that this gut commensal unexpectedly produces an isoform of α-galactosylceramide, a sponge-derived sphingolipid that is the prototypic ligand for the host immune receptor CD1d.

Results and Discussion

Bioinformatic Insights into Bacteroides fragilis Sphingolipid Biosynthesis

To gain insight into the potential role of Bacteroides sphingolipids in mediating microbiota–host interactions, we set out to define the
**Author Summary**

While human gut bacteria are thought to produce diffusible molecules that influence host biology, few of these molecules have been identified. Species of *Bacteroides*, a Gram-negative bacterial genus whose members often comprise >50% of the gut community, are unusual in that they produce sphingolipids, signaling molecules that play a key role in modulating the host immune response. Sphingolipid production is ubiquitous among eukaryotes but present in only a few bacterial genera. We set out to construct a *Bacteroides* strain that is incapable of producing sphingolipids, knocking out a gene predicted to encode the first enzymatic step in the *Bacteroides* sphingolipid biosynthetic pathway. The resulting mutant is indeed deficient in sphingolipid production, and we purified and solved the structures of three sphingolipids that are present in the wild-type strain but absent in the mutant. To our surprise, one of these molecules is a close chemical relative of a sponge sphingolipid that is the prototypical ligand for a host receptor that controls the activity of natural killer T cells. Like the sponge sphingolipid, the *Bacteroides* sphingolipid can modulate natural killer T cell activity, suggesting a novel mechanism by which *Bacteroides* in the gut might influence the host immune response.

![Chemical structures of the *B. fragilis* sphingolipids and related molecules.](image)

**Figure 1.** Chemical structures of the *B. fragilis* sphingolipids and related molecules. (A) *B. fragilis* produces the phosphosphingolipid ceramide phosphorylethanolamine (CPE, top) and the corresponding free ceramide (ceramide_{Eh}, middle), which are similar in structure to the most abundant (4,5-dehydro) and third-most abundant (4,5-dihydro) forms of sphingomyelin in human plasma (bottom). (B) *B. fragilis* produces the glycosphingolipid α-galactosylceramide (α-GalCer_{Bf}, top), which is similar in structure to the sponge-derived α-galactosylceramide agelasphin-9b (middle) and a widely used derivative of agelasphin-9b, KRN7000 (bottom). Chemical groups that vary among the molecules in each column are colored red and blue for *B. fragilis* and non-*B. fragilis* sphingolipids, respectively. CPE, ceramide_{Eh}, and α-GalCer_{Bf} were each purified as inseparable mixtures of varying lipid chain length. The proposed structures of the most abundant species are shown here. doi:10.1371/journal.pbio.1001610.g001

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essential for Bacteroides viability. An interesting alternative comes from the observation that dihydrophosphoglycerine, the putative substrate of BF2462, is toxic to Bacteroides melaninogenicus at 4 μM [11]; the absence of BF2462 could therefore lead to the buildup of a toxic intermediate.

Nevertheless, since the yeast homolog of BF2461 constitutes the entry point to the sphingolipid pathway, we hypothesized that the Δ2461 mutant would be sphingolipid-deficient, providing an ideal starting point for enumerating the B. fragilis sphingolipids. To test our hypothesis, we used comparative HPLC-ELSD to analyze alkaline-stable lipid extracts from the wild-type (WT) and Δ2461 strains. Our analysis revealed three major peaks that were present in the WT but not the Δ2461 extract (Figure 2).

Preparative thin layer chromatography was used to purify multimilligram quantities of these compounds, and HPLC-MS analysis of the purified material revealed that each peak consists of a mixture of co-migrating compounds that vary in mass by 14 Da. Measured in negative mode, the most abundant mass ions for peaks 1, 2, and 3 were 677.5 Da, 554.5 Da, and 716.6 Da, respectively.

**Elucidating the Structures of the B. fragilis Sphingolipids**

To solve the chemical structures of the sphingolipid species, we first subjected the purified compounds to high-resolution MS. The mass of peak 1 was consistent with a ceramide phosphorylthanolamine (CPE) (C27H32N6O12P; [M-H]− m/z calculated 554.5234, observed 557.5221), a sphingomyelin isofrom previously found to be the principal B. fragilis sphingolipid, while the mass of peak 2 was consistent with the corresponding dihydroceramide base (C34H62NO9; [M-H]− m/z calculated 716.5418, observed 716.5420) (Figure 1A; Figure S1 in Supporting Information S1). A set of 1D and 2D NMR experiments on the purified compounds from peaks 1 and 2 yielded resonances and couplings consistent with these assignments (see S4.1 and S4.3 in Supporting Information S1).

**B. fragilis Produces α-Galactosylceramide**

In contrast, peak 3 was not a known compound. High-resolution MS analysis of the purified material from peak 3 was consistent with an empirical formula of C34H68NO4 (C34H68NO4; [M-H]− m/z calculated 677.5234, observed 677.5221), a sphingomyelin isofrom previously found to be the principal B. fragilis sphingolipid, while the mass of peak 2 was consistent with the corresponding dihydroceramide base (C34H68NO4; [M-H]− m/z calculated 716.5418, observed 716.5420) (Figure 1A; Figure S1 in Supporting Information S1). A set of 1D and 2D NMR experiments on the purified compounds from peaks 1 and 2 yielded resonances and couplings consistent with these assignments (see S4.1 and S4.3 in Supporting Information S1).

α-GalCer_Bf for 13 d and assessed the presence of CD3+ mononuclear cells (PBMCs) from six independent donors with staining was ligand- and TCR-specific (Figure 3A; Figure S2 in Supporting Information S1), indicating that the tetramer preparation, Yadav and Bluestone), indicating that the tetramer staining was ligand- and TCR-specific (Figure 3A; Figure S2 in Supporting Information S1). The iNKT cell hybridomas tested produced IL-2 in response to both the marine-sponge-derived and B. fragilis-derived sphingolipids in a dose-dependent manner and in absence of antigen presenting cells (APCs). These results suggested that α-GalCer_Bf is a stimulatory ligand that directly activates iNKT cells in vitro (Figure 3B–C; Figure S3 in Supporting Information S1).

We next examined the ability of purified α-GalCer_Bf to stimulate freshly isolated mouse and human iNKT cells in vitro. Liver mononuclear cells, 30%–50% of which are NKT cells, were incubated with splenocytes as APCs in the presence of increasing doses of α-GalCer_Bf and examined for IFN-γ production. α-GalCer_Bf induced IFN-γ in a dose-dependent and CD1d-dependent manner. The response was inhibited completely by anti-CD1d antibodies (Figure 3D), consistent with our previous result that NKT cell stimulation required ligand presentation by CD1d (Figure 3B).

To explore whether the response of NKT cells to α-GalCer_Bf is conserved in humans, we determined whether Vα24+ cells could be expanded in vitro with purified α-GalCer_Bf as previously described for KRN7000 [31]. We cultured peripheral blood mononuclear cells (PBMCs) from six independent donors with 0.1 μg/ml KRN7000, 1 μg/ml α-GalCer_Bf or 1 μg/ml ceramide_Bf for 13 d and assessed the presence of CD3+Vα24+ cells by...
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[Ceramide phosphorylethanolamine]

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A. total ion count (x10⁶) vs. elution time (min)

B. total ion count (x10⁶) vs. elution time (min)

C. ceramide phosphorylethanolamine

D. Ceramide phosphorylethanolamine

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Figure 2. *B. fragilis* BF2461 is deficient in the production of sphingolipids. HPLC-MS traces of crude lipid extracts of (A) wild-type *B. fragilis* and (B) the sphingolipid-deficient mutant BF2461 are shown. The traces shown are the total ion count (black) and the extracted ion traces of sphingolipid masses for ceramide (m/z [M+H]: 540.5, 554.5, 568.5, 582.6; green), CPE (m/z [M+H]: 663.5, 677.5, 691.5, 705.5; brown), α-GalCerBf (m/z [M+H]: 702.6, 716.6, 730.6, 744.6; blue), and phosphatidylethanolamine (m/z [M+H]: 648.5, 662.5, 676.5, 690.5). Peaks corresponding to the three sphingolipids, but not the phospholipid phosphatidylethanolamine, are absent in *B. fragilis* BF2461. (C) High-resolution mass spectra of CPE, ceramideBf, and α-GalCerBf collected in the negative ion mode. The insets show a zoomed-in view of the dominant field of peaks for each compound. (D) A table showing the calculated and observed masses for the dominant mass ions for each compound. See S1.1 in Supporting Information S1 for details.
observation of dihydroceramide production by *B. fragilis*. On the basis of these observations, we propose a model of *Bacteroides* sphingolipid biosynthesis that closely mirrors the eukaryotic pathway (Figure 4). Given that sphingolipids comprise ~30% of total cellular lipids and *Bacteroides* lacks an endoplasmic reticulum (the site of eukaryotic sphingolipid synthesis), the regulation of this pathway in the context of lipid metabolism and the localization of its biosynthetic enzymes will be important areas to explore.

**Figure 3. α-GalCerBf binds CD1d and activates NKT cells.** (A) Hybridomas were stained with anti-CD3 antibodies and empty mCD1d tetramers or CD1d tetramers loaded with α-GalCerBf or KRN7000. Flow cytometry plots are pregated on DAPI− events in lymphocyte gate stained with CD3 antibodies and the specified tetramer. Plots representative of three independent experiments are shown. (B) Hybridomas were cultured with BMDCs pre-pulsed with LPS or LPS + α-GalCerBf in the presence of control Ig or anti-CD1d blocking antibodies. IL-2 secretion was measured in supernatants 16 h later. Data are representative of three independent experiments. (C) Plates were coated with CD1d monomers and loaded with the specified amounts of α-GalCerBf. Hybridomas were then incubated for 16–18 h and IL-2 was measured in the supernatants by ELISA. Data are representative of three independent experiments. (D) Liver mononuclear cells were cultured with splenocytes plus increasing amounts of α-GalCerBf in the presence or absence of anti-CD1d blocking antibodies. IFN-γ secretion was measured in supernatants on day 5. Data are representative of three independent experiments. (E and F) Representative flow cytometry plots and pooled data of PBMCs cultured for 13–14 d with 0.1 μg/ml KRN7000, 1 μg/ml α-GalCerBf, or 1 μg/ml ceramideBf. Dot plots show all events in the lymphocyte gate stained with 6B11 (specific for Vα24) and CD3 antibodies. Gate shows percentage of Vα24+CD3+NKT cells pre- and postexpansion. Pooled data showing six individual donors tested in three independent experiments. *p = 0.0078, **p = 0.0020 compared to control day 13 culture. (G–I) Bone-marrow-derived dendritic cells were pulsed in vitro with LPS only or LPS + α-GalCerBf for 24 h. The 0.4 × 10^6 cells were transferred to WT mice, which were treated with control Ig or anti-CD1d blocking antibody prior to cell transfer. Liver mononuclear cells were analyzed 16–18 h later. Data shown were pooled from three independent experiments. (G) Expression of CD25 and CD69 on gated CD3+tetramer+ cells. Representative flow cytometry plots and pooled data showing fold change of CD25 and CD69 surface expression compared to NKT cells isolated from mice transferred with LPS-pulsed BMDCs. (H) Representative flow cytometry plots and pooled data of intracellular IFN-γ expression on gated CD3+tetramer+ cells. (I) Serum IFN-γ levels.
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Materials and Methods

Detailed methods are provided in Supporting Information S1.

Construction of Mutant Strain ΔBF2461

Primer sequences are listed in Table S1 in Supporting Information S1. DNA fragments flanking BF2461 were PCR amplified from B. fragilis NCTC9343 using the following primers: LF_5'; LF_3'; RF_5'; RF_3'. These fragments were digested with SstI and MluI and cloned into the SstI site of pNJR6. The resulting plasmid was introduced into B. fragilis NCTC9343 by conjugation, and cointegrates were selected using erythromycin. Cointegrates were passaged, plated on nonselective medium, and replica plated to medium containing erythromycin. Erythromycin-sensitive colonies were screened by PCR to detect those acquiring the mutant genotype.

Purification of α-GalCerBf

B. fragilis NCTC9343 was cultured under standard conditions, and harvested cells were extracted with CHCl₃:MeOH (2:1). The organic extract was subjected to alkaline hydrolysis, neutralized, and extracted with CHCl₃:MeOH (2:1). The crude extract was purified by preparative TLC (CHCl₃:MeOH:H₂O, 65:25:4) to give α-GalCerBf (Rf = 0.6). For complete experimental details, including yields and full characterization (NMR, high-resolution mass spectrometry) of all compounds, see Supporting Information S1. α-GalCerBf was isolated in five independent batches, and the in vitro and in vivo experiments were repeated with different batches of purified compound.

α-GalCerBf Used for Immunological Experiments

α-GalCerBf, CPE, and the ceramide base were each purified as an inseparable mixture of varying lipid chain length. Mass spec analysis of the methanolyzed long chain base (LCB) (S4.6 in Supporting Information S1) suggests that this portion of the structure carries the variation (see next paragraph). The inseparable mixture of alpha-galactoslyceramides (>95% pure), referred to as “purified α-GalCerBf,” was the material used for the immunological experiments.

Figure 4. Proposed pathway for Bacteroides sphingolipid biosynthesis. BF2461, a putative serine palmitoyltransferase, would catalyze the pyridoxal-phosphate-dependent conjugation of serine and a long-chain acyl-CoA to form 3-ketodihydrosphingosine, which would undergo a ketoreductase-catalyzed conversion to dihydrosphingosine. At this branchpoint, dihydrosphingosine could either be phosphorylated by the putative sphingosine kinase BF2462 to form S1P, or it could undergo N-acylation to yield the observed dihydroceramide intermediate (compound 2). This common C₃₄ scaffold would then be the substrate for two alternative head group modifications: glycosylation to form α-GalCerBf, or phosphorylethanolamine group transfer to form CPE.

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Analysis of Lipid Tail Length Variation

Methanolysis of ceramide Dβ produced a mixture of three LCB amines that could be separated and analyzed by HPLC-MS (S4.6 in Supporting Information S1). Analysis of each by HRMS indicated that they are structural variants that differ in tail chain length. These data suggest the major parent α-GalCerBf variants (m/z 716.57, m/z 730.58, and m/z 744.60) also differ in chain length of the LCB.

Hybridoma Stimulation

For dose titration experiments, BMDCs and DN3A1-1.2 and N30-2C12 NKT hybridomas (M. Kronenberg) and GFP36 CD4+ hybridoma were cultured at a 3:1 hybridoma:BMDC ratio and the indicated doses of KRN7000 or α-GalCerBf in the presence of 1 μg/ml LPS. Supernatants were harvested after 24 h and IL-2 production was measured by ELISA. For APC-free experiments, CD1d monomers were coated on a 96-well plate for 1 h, and wells were blocked with PBS/10% FBS. The indicated amount of α-GalCerBf was added to each well and incubated at 37°C for 3 h. After washing unbound α-GalCerBf, hybridomas were added. Supernatants were harvested after 16–18 h and IL-2 production was measured by ELISA. For in vitro CD1d blocking experiments, α-GalCerBf pulsed BMDCs were cultured at a 3:1 hybridoma:BMDC ratio in the presence of 10 μg/mL anti-CD1d antibody (Clone 1B1, BD Pharmingen). Supernatants were harvested after 16–18 h and IL-2 production was measured by ELISA.

In Vitro Stimulation of Human NKT Cells

For blood draws from healthy donors, informed consent was obtained in accordance with approved University of California, San Francisco IRB policies and procedures (IRB 10-02596). PBMCs were cultured for 13–14 d in RPMI containing 10% autologous serum plus lipids as described in Figure 3. On day 1 of culture, 100 U/ml IL-2 was added. Cultures were harvested on day 13 or 14 and the percentage of CD3+NKT cells was determined by flow cytometry after staining with CD3 and 6B11 antibodies.

In Vivo Activation of NKT Cells

Mice were sacrificed 16–18 h after transfer of 0.4×10⁶ mature CD86hiMHCIIhi BMDCs. Livers were cut into small pieces and harvested after 16–18 h and IL-2 production was measured by ELISA. For blood draws from healthy donors, informed consent was obtained in accordance with approved University of California, San Francisco IRB policies and procedures (IRB 10-02596). PBMCs were cultured for 13–14 d in RPMI containing 10% autologous serum plus lipids as described in Figure 3. On day 1 of culture, 100 U/ml IL-2 was added. Cultures were harvested on day 13 or 14 and the percentage of CD3+NKT cells was determined by flow cytometry after staining with CD3 and 6B11 antibodies.

References

4. LaBach JP, White DC (1969) Identification of ceramide phosphorylethanola-


