Membrane sphingolipids as essential molecular signals for *Bacteroides* survival in the intestine

Dingding An[a,b], Chongzheng Na[c], Jacek Bielawski[d], Yusuf A. Hannun[d], and Dennis L. Kasper[a,b,1]

[a]Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, 02115; [b]Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115; [c]Department of Civil Engineering and Geological Sciences, University of Notre Dame, Notre Dame, IN, 46556; and [d]Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, SC 29425

As predominant intestinal symbiotic bacteria, *Bacteroides* are essential in maintaining the health of the normal mammalian host; in return, the host provides a niche with plentiful nutrients for the symbionts. However, the intestinal environment is replete with chemical, physical, and biological challenges that require mechanisms for prompt and adept sensing of and responses to stress if the bacteria are to survive. Herein we propose that to persist in the intestine *Bacteroides* take advantage of their unusual bacterial sphingolipids to mediate signaling pathways previously known to be available only to higher organisms. Sphingolipids convey diverse signal transduction and stress response pathways and have profound physiological impacts demonstrated in a variety of eukaryotic cell types. We propose a mechanism by which the formation of specific sphingolipid membrane microdomains initiates signaling cascades that facilitate survival strategies within the bacteria. Our preliminary data suggest that sphingolipid signaling plays an important role in *Bacteroides* physiology, enabling these bacteria to persist in the intestine and to perform other functions related to symbiosis.

stress response | bacterial sphingolipids | lipid rafts

The *Bacteroides* species make up a predominant genus of bacteria residing within the mammalian intestine. Organisms belonging to this genus have been the subject of extensive research and possess many distinctive features contributing to their ability to flourish in the intestine. For example, *Bacteroides* have sophisticated ability to degrade polysaccharides (for energy acquisition) (1), pronounced phase variation in surface polysaccharide synthesis (for antigenic versatility) (2, 3), and extensive surface decoration with host glycans (for molecular mimicry) (4). These bacteria display another extremely rare structural feature that has been recognized for decades but remains largely unstudied: membrane sphingolipids. Sphingolipids were generally thought to exist exclusively in eukaryotes until they were discovered in a handful of bacterial and viral lineages (5, 6). What is the function of sphingolipids in bacteria? Are they important in maintaining *Bacteroides* in their ecological niches? Herein we provide first evidence supporting an important role for sphingolipids in the ability of *Bacteroides* to survive stress, and we discuss the potential role of sphingolipids in maintaining intestinal symbiotic bacterial populations.

Results and Discussion

Are Sphingolipids Important for *Bacteroides* Growth? Sphingolipids are characterized by an aliphatic amino alcohol sphingoid backbone called long-chain base (LCB). LCB is attached via an amide bond to a fatty acid and via an ester linkage to a polar head group (Fig. 1*A*). Sphingolipids are always present in eukaryotic cells but are absent in most bacteria, whose membranes comprise only glycerol-based phospholipids (Fig. 1*B*). However, a few bacterial species possess both phospholipids and sphingolipids. These sphingolipid-containing bacteria are highly represented by genera in the phylum Bacteroidetes, including gut-associated *Bacteroides* (5, 7), oral cavity–associated *Porphyromonas* (8), gut and oral

cavity–associated *Prevotella* (9), and soil-associated *Sphingobacterium* (10). Other prokaryotes constituted with sphingolipids include the genus *Sphingomonas* in the class Alphaproteobacterium, *Bdellovibrio* species in the class Deltaproteobacterium, and *Fusobacterium* species (11). This is by no means a complete list, and it is very likely additional bacterial species contain sphingolipids, especially in the Bacteroidetes phylum. Apart from the fact that the glycosphingolipids in *Sphingomonas* are potent antigens in the activation of invariant natural killer T cell populations during infection (12, 13), little is known about bacterial sphingolipid function.

To determine whether sphingolipids are essential for maintaining cell integrity and normal growth, we studied the model organism *Bacteroides fragilis*, which is prevalent in the intestine and is of substantial clinical significance. In conducting this work, we took advantage of years of research on eukaryotic sphingolipids and employed myriocin, a widely used, potent, specific inhibitor of sphingolipid biosynthesis, to create sphingolipid-deficient *B. fragilis* cultures. Although genetic approaches (e.g., gene knockout and complementation) are necessary to fully address the function of sphingolipids, chemical methods have the advantage of producing fast and reliable results in initial studies. In the eukaryotic sphingolipid biosynthetic pathway (14, 15) depicted in Fig. 1*C*, myriocin chemically knocks out sphingolipid synthesis by disrupting the activity of serine palmitoyl transferase (SPT), the first enzyme of committed sphingolipid synthesis (16, 17) that condenses palmitoyl-CoA and serine to 3-ketosphinganine. The genome of wild-type *B. fragilis* strain NCTC 9343 has an SPT ortholog (encoded by BF2461) with a high degree of homology to the human SPT enzyme subunits SPTLC1, SPTLC2, and SPTLC3 [E values: $\leq E^{-44}$ by standard BLASTP search (18); http://blast.ncbi.nlm.nih.gov]. In addition, SPT orthologs have been reported in several other sphingolipid-producing bacteria (19, 20) and are well conserved in *Bacteroides* and related species (Table S1). In the following steps, 3-ketosphinganine is converted to sphinganine by 3-ketosphinganine reductase, and sphinganine is further transformed to dihydroceramide by ceramide synthase. Dihydroceramide and its modifications (such as ceramide with a double bond between C4 and C5 on LCB and phytoceramide with an extra hydroxyl group at C5 on LCB) are the central precursors for more complex sphingolipids and themselves are often important components of membrane lipids. Because myriocin
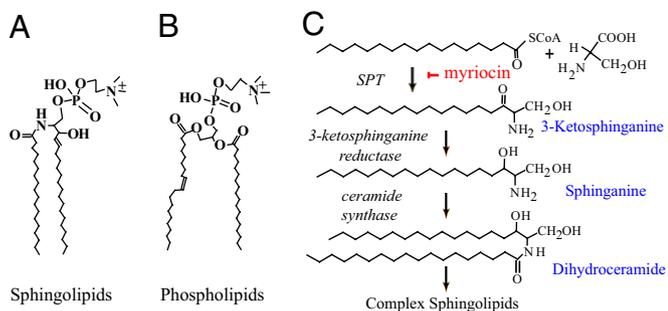
Fig. 1. Typical structures of (*A*) sphingolipids and (*B*) phospholipids. (*C*) Initial steps in eukaryotic sphingolipid biosynthetic pathway.

inhibition is specific to SPT but not other enzymes in the pathway, the quantity of ceramide-like molecules can be regarded as an indicator of the entire sphingolipid population in cells. By using electrospray ionization tandem mass spectrometry (ESI/MS/MS) (21), we found that addition of 5 μM myriocin to *B. fragilis* can chemically inhibit more than two thirds of ceramide-like structures (Figs. S1, S2 and S3). Thus the myriocin-treated *B. fragilis* cells are sphingolipid-deficient.

An overnight culture of the *B. fragilis* wild-type strain was diluted 1:1,000 in fresh rich medium (recipe in *SI Materials and Methods*) in the presence or absence of 5 μM myriocin, and the diluted cultures were incubated under anaerobic conditions at 37 °C in test tubes. Optical density at 650 nm (OD_{650}) and colony-forming units (CFUs) per milliliter were monitored as measures of bacterial growth and death. The growth curves in Fig. 2 show that inhibition of sphingolipid synthesis resulted in no *B. fragilis* growth defect; the two cultures had analogous growth kinetics in the log phase and similar cell densities in the early stationary phase. However, with the sphingolipid-deficient culture was completely wiped out within 60 h after entering the stationary phase, whereas ≈4×10^5 CFUs/mL of the sphingolipid-proficient cells were still alive at the same time point. These results suggest that sphingolipids probably are not essential for the active growth of *B. fragilis* but are involved in long-term survival of cells in the stressful stationary phase.

Are Sphingolipids Important in the *Bacteroides* Stress Response? Seeking further evidence that *B. fragilis* sphingolipids are important for bacterial stress survival, we applied common types of

stress encountered by intestinal microbes to stationary-phase cells grown in the presence or absence of myriocin, and we then monitored cell numbers. The stress challenge consisted of heat shock accompanied by oxidative stress whereby cultures were incubated at 42 °C in air for 14 h. Although cell death was significant in both cultures after such treatment, bacterial survival in the sphingolipid-deficient culture was approximately 2 logs lower than that in the sphingolipid-proficient culture (Fig. 3). Thus *Bacteroides* sphingolipids seem to play an important role in bacterial survival under stress.

Can the Stress Response Be Mediated by Sphingolipid Signaling in *Bacteroides*? Sphingolipids, mostly enriched in the outer leaflet of the eukaryotic plasma membrane as well as in endosomes, are key signal transduction molecules with high specificity in response to various cell stresses, such as cytokines, growth factors, death factors, heat, and UV radiation (15, 22). The biological outcomes of sphingolipid signaling are profound and diverse, including cell apoptosis, proliferation, differentiation, mitogenesis, and migration; angiogenesis; and inflammation. Cholesterol is indispensable for sphingolipid signaling in mammals, facilitating formation of sphingolipid-enriched platforms ("lipid rafts") in membranes to function as membrane-reactive centers. In the mammalian intestine, *Bacteroides* species have access to abundant cholesterol—both provided by food intake and secreted by the host (23). Therefore, under stressful conditions, *Bacteroides* have both sphingolipids and cholesterol at their disposal to assemble a unique stress-response signaling mechanism previously known to be available only to higher organisms.

If *Bacteroides* use a eukaryotic-like sphingolipid- and cholesterol-dependent mechanism to respond to stress, this mechanism will be most efficient only when both sphingolipids and cholesterol are present. To test this hypothesis, we prepared sphingolipid-proficient cultures with and without cholesterol (*Sph+Chol+* and *Sph+Chol−*) and sphingolipid-deficient cultures with and without cholesterol (*Sph−Chol+* and *Sph−Cho−*). Because the rich medium used in the studies depicted in Figs. 2 and 3 contains a yeast product (ergosterol) that can functionally substitute for cholesterol in sphingolipid signaling (24), we prepared the cholesterol-free cultures in a glucose-supplemented minimal medium (recipe in *SI Materials and Methods*). Cholesterol (30 μg/mL) was added to formulate cholesterol-rich cultures; the addition of cholesterol at this concentration had no remarkable impact on growth. Moreover, we purposefully chose a distinct stress type—
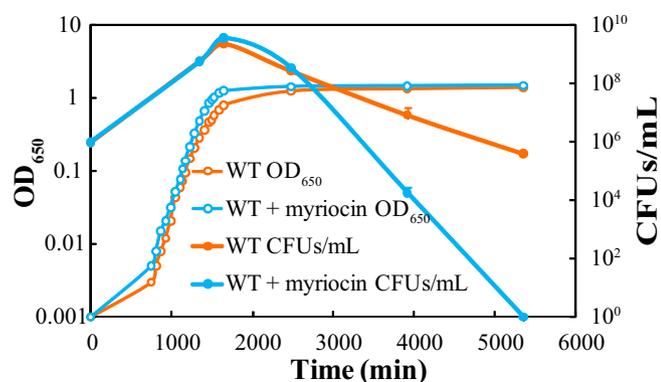

Fig. 2. Sphingolipids affect the survival of *Bacteroides* in stationary phase but not the growth of the bacteria in log phase. Growth curves are shown for the wild-type *B. fragilis* culture in which sphingolipids were intact (WT) and the myriocin-treated *B. fragilis* culture in which sphingolipid synthesis was inhibited (WT + myriocin). Optical density curves are representative of three replications. Error bars for CFUs/mL curves show the SD of triplicate samples in one representative experiment.
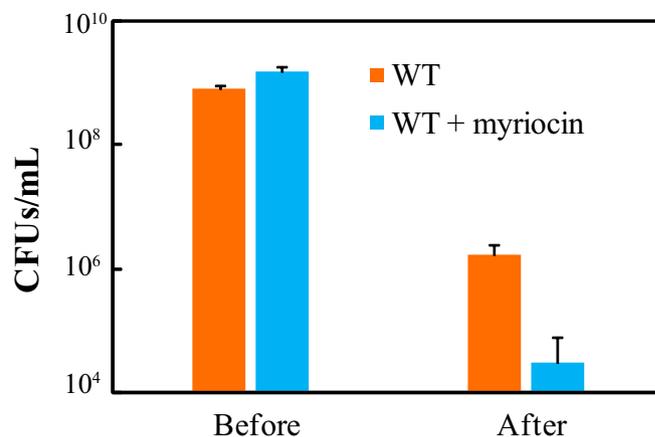

Fig. 3. Sphingolipids are important in the response of *Bacteroides* to stress. Rich-culture cells proficient with sphingolipids (WT) were compared with those deficient in sphingolipids (WT + myriocin) in terms of survival under heat shock and oxidative stress. Error bars show the SD of triplicate samples in one representative experiment.
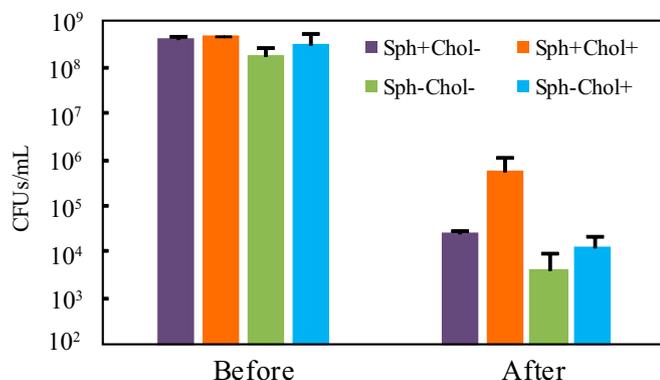
Fig. 4. Sphingolipids and cholesterol are both needed for an efficient stress response. Cells grown in minimal medium with different combinations of sphingolipids and cholesterol were compared in terms of survival after DNA crosslinking and oxidative stress. Error bars show the SD of duplicate samples in one representative experiment.

DNA crosslinking—in this experiment. DNA crosslinking is common and can be induced in living organisms by both exogenous and endogenous agents, such as alkylating agents, nitrous acids, and other reactive species (25). Mitomycin C—a potent chemotherapeutic compound commonly used to treat a broad range of tumors, including gastrointestinal cancers (26)—served as the crosslinking agent here. When both sphingolipids and cholesterol were available to *B. fragilis*, the survival rate was the highest, with more than 1.4 logs more cells alive than in any of the other three cultures (Fig. 4). Deficiency of either cholesterol or sphingolipids caused a lower survival rate, and loss of both only made the bacteria slightly less viable than loss of either, suggesting cholesterol and sphingolipids function by related mechanisms. Using a different stress challenge under different growth conditions, this experiment confirmed the conclusion suggested by data in Fig. 3 and proposed that *Bacteroides* organisms probably use the eukaryotic-like sphingolipid signaling mechanism for various stress survival.

Is Sphingolipid Signaling Feasible in *Bacteroides*? From a genomic viewpoint, conservation of SPT in sphingolipid-producing bacteria demonstrates that these bacteria are committed to sphingolipid metabolism and suggests that they may share other enzymes in the sphingolipid biosynthetic pathway (Fig. 1*C*) with eukaryotes. However, a standard BLASTP search in *Bacteroides* genomes does not return dependable 3-ketosphinganine reductase and ceramide synthase homologs. Another important class of enzymes is sphingomyelinase, which degrades sphingomyelin to ceramide. *Bacteroides* largely lack these enzymes, although they are found in many pathogens [e.g., *Staphylococcus aureus* (27)]. It is possible that *Bacteroides* have structural homologs to these enzymes, which would not be revealed by standard BLASTP searches. Nonetheless, complex and simple sphingolipid molecules are detected in cultures of *Bacteroides* species. For example, in addition to our data in Fig. S1, it was reported that as much as 10% of total lipid content was (dihydro)ceramide in *B. fragilis* (7). The discrepancy between genomic predictions and chemical analyses indicates either that a more in-depth search of the genome is needed or that bacteria and eukaryotes use different pathways to synthesize sphingolipids.

Several bacterial sphingolipid structures have been characterized (7–9, 28). In bacteria, both LCBs and fatty acid acyl chains are typically saturated with 17–19 carbons in length (dihydroceramide- or phytoceramide-based) and sometimes the fatty acids have an extra 3-hydroxyl group. They are also usually branched with a methyl group in either the iso or the anteiso position. These characteristics are different from those of eukaryotic sphingolipids, which usually have a double bond between C4 and C5 on the LCBs (ceramide-

based) and have unbranched acyl chains 18–20 carbons in length. Another notable feature is the polar head group. Eukaryotes most often have phosphorylcholine as the head group to form sphingomyelin, whereas bacteria are largely devoid of sphingomyelin but instead characteristically have ceramide phosphorylethanolamine (CPE), ceramide phosphorylglycerol (CPG), ceramide phosphoryl-myo-inositol, ceramide phosphorylmannose, and other complex glycosphingolipids (5, 10, 29–31). The major sphingolipid species in *B. fragilis* include CPE and CPG. These structural analyses suggest that bacterial sphingolipids largely resemble their eukaryotic counterparts and probably behave similarly in cell membrane bilayers.

Because of differences in chemical structures and physical properties, phospholipids and sphingolipids form separate domains in model membranes (32). The distinction between the two membrane domains is strengthened by the presence of cholesterol or cholesterol-like lipid molecules in eukaryotic cells. The heterogeneity in membrane structure is thought to provide a physical and chemical basis for sphingolipid signaling. Although this topic is controversial and unresolved in many respects, a popular view is that membrane signaling is associated with the sphingolipid domains, which are also enriched in cholesterol and specific proteins. These domains are more tightly packed and have a higher melting temperature because of the highly hydrophobic interactions of the sphingolipid acyl chains with cholesterol and the extensive hydrogen bonding facilitated by the amide linkage and the hydroxyl group. Sphingolipid-enriched domains are thus in the liquid-ordered phase and have greater lateral mobility than the surrounding liquid-disordered phospholipid domains (Fig. 5*A*). When signaling is triggered by stresses, considerable ceramide is
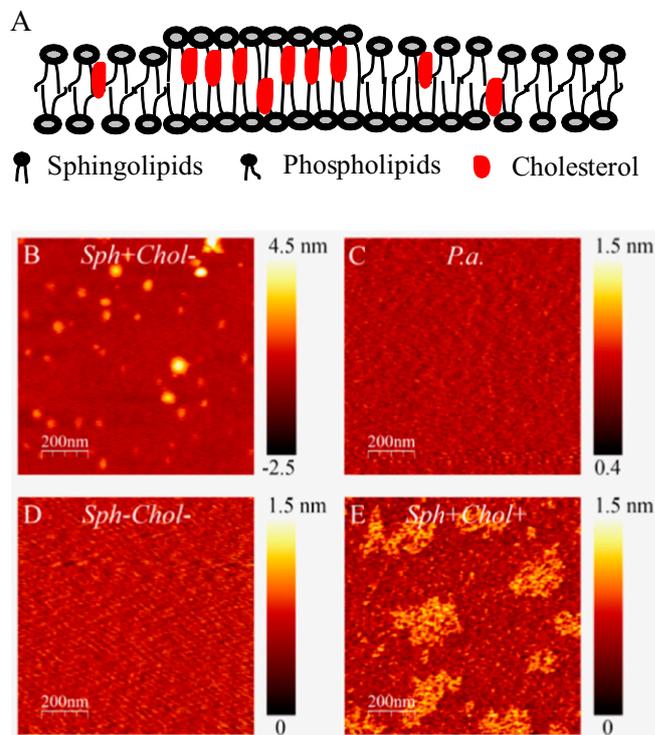
Fig. 5. *Bacteroides* lipids form sphingolipid- and cholesterol-dependent heterogeneous domains in supported bilayers. (*A*) Schematic sketch shows formation of heterogeneous membrane domains. (*B–E*) Representative AFM height images (1 μm × 1 μm) of reconstituted bilayers using lipid extractions from *B. fragilis* Sph+Chol– (*B*), *P. aeruginosa* (*P.a.*; *C*), *B. fragilis* Sph–Chol– (*D*), and *B. fragilis* Sph+Chol+ (*E*). Color bars along AFM images indicate height ranges. The extended height range in sample *Sph+Chol–* was due to assembly of multiple lipid bilayers instead of single bilayers. Areas of multiple lipid bilayers were excluded from calculations shown in text.

generated through sphingomyelin hydrolysis or de novo synthesis (33–35). The increase in ceramide can significantly promote the liquid-ordered state and result in merging of adjacent microdomains and formation of bigger platforms. These spatially organized platforms can effectively concentrate and recruit specific proteins, generate signaling molecules, and initiate downstream events (36–39). In this process, sphingolipids are thought to be important in two ways: they are the central components of the bioactive membrane domains, and they can be signaling molecules themselves.

If *Bacteroides* organisms are capable of sphingolipid-mediated signaling, the lipid membrane presumably will have distinct microdomains. Atomic force microscopy (AFM), a widely used method, has shown that eukaryotic phospholipid and sphingolipid species can form separate domains when these lipids are mixed and reconstituted in lipid bilayers on inert surfaces (40). The two domains are characterized by two discrete phases with a small variation (<2 nm) in the "height image." Of these two phases, sphingolipid domains are taller. Furthermore, AFM clearly shows the impact of cholesterol, revealing that its addition considerably enlarges the microdomains. To investigate whether such heterogeneity exists in *Bacteroides* lipid bilayers, we extracted lipids from stationary-phase *B. fragilis* cultures grown under several conditions. Each lipid sample was then reconstituted on a mica surface to form bilayers and was inspected by AFM.

We first grew *B. fragilis* cells in minimal medium without exogenously added cholesterol (*Sph+Chol−*). After reconstitution in bilayers, a two-phase pattern was observed by AFM. These two phases represented phospholipid- and sphingolipid-enriched domains with an average height difference of 0.5 nm. The average size (diameter) of the taller sphingolipid domain was 44 ± 15 nm (Fig. 5B). We next applied the same procedure to a lipid preparation from *Pseudomonas aeruginosa*, a bacterium that produces phospholipids but not sphingolipids. Serving as a negative control, lipids in this sample were homogeneously distributed, with one single height and phase across the examined surface (Fig. 5C). To investigate whether the heterogeneity shown in Fig. 5B was due to the presence of sphingolipids, we prepared lipids from myriocin-treated *B. fragilis* culture (*Sph−Chol−*) and found that the extracted lipids became homogeneously distributed on the surface bilayer in a manner resembling that seen with the *P. aeruginosa* sample (Fig. 5D). Finally, to examine whether the presence of cholesterol changes lipid domain organization, we extracted lipids from a *B. fragilis* culture grown in the presence of 30 μg/mL cholesterol (*Sph+Chol+*). As predicted, the sphingolipid domains were considerably enlarged, with an average size of ≈168 ± 45 nm (Fig. 5E). The average height difference of the two phases in this sample was 0.3 nm. These observations provide strong evidence that the lipid bilayer organization in *Bacteroides* is characterized by heterogeneity and bears a resemblance to that of eukaryotic plasma membrane lipids. Therefore, from a physicochemical point of view, *Bacteroides* sphingolipids can form membrane functional units for signaling.

Proteomic studies from the literature provide additional information suggesting the existence of bacterial sphingolipid microdomains. Proteins with an SPFH (stomatin, prohibitin, flotillin, HflK/C) domain play important roles in scaffolding sphingolipid-enriched, detergent-resistant eukaryotic membrane microdomains and have become the hallmarks of the "lipid raft" (41). Remarkably, prokaryotic SPFH orthologs are widely distributed in the phylogenetic tree, including *Bacteroides* species, and are proposed as the origins of their eukaryotic counterparts (42). It is tempting to speculate that *Bacteroides* SPFH orthologs interact with sphingolipids and cholesterol to facilitate the formation of signaling microdomains. Moreover, the presence of SPFH orthologs in bacterial species that do not produce sphingolipids is intriguing. It is well known that bacterial phospholipids have highly diverse structures generated by lipid homeostasis mechanisms

(43). One hypothesis is that these SPFH orthologs interact with a subset of phospholipids to form special functional membrane units for signal transduction. Confirmation of this hypothesis would indicate that membrane heterogeneity is a common theme in all bacteria.

Does *Bacteroides* Sphingolipid Signaling Have Ecological Significance? Sphingolipid signaling in eukaryotes is an exceedingly vibrant process characterized by a high turnover rate of lipid species and extremely versatile signaling functions (22, 44, 45). The magnitude of the complexity has just begun to be elucidated, but what is already known suggests that eukaryotic cells craft an enormously dynamic environment for themselves, commanding signaling processes with a high degree of sophistication. As single-celled prokaryotes, bacteria usually do not encounter such fierce challenges except if their survival depends on close association with eukaryotic hosts as in the case of intestinal symbionts. These symbiotic microbes thus are under tremendous selection pressure and must deal efficiently with the ever-changing host environment.

Born sterile, mammals become colonized with microbes immediately after birth, at which point their life-long relationship with microbial partners (the microbiota) commences. The colonization of many sites in and on mammals is most strikingly exemplified in the large intestine, where the density of microbial organisms can be as high as 10^{12}/g luminal content. This intense colonization evolves into a mutually beneficial relationship. In exchange for niches with the right growth conditions, the microbiota endows mammals with abilities they have not developed on their own. Consequently, symbiotic bacteria are implicated in a number of major health issues. In a sense, the intestinal microbiota serves as an acquired multifunctional eukaryotic organ (46–48).

As the "acquired organ" develops postnatally, the intestinal microbiota is continuously challenged by the host environment. Along with the normal peristaltic propulsion of the intestine, the renewal of intestinal epithelial cells at estimated rates of 20–50 million and 2–5 million cells per minute in the small intestine and the colon, respectively (49), constantly generates a fresh interface between epithelial cells and microbial cells; these changes require immediate recognition and tolerance. In addition, thermal instability results from fluctuations in the local intestinal temperature in response to various cues, including food intake, inflammation, pharmacologic agents, or emotional stresses. Moreover, the intestine presents microbes with an extraordinarily complex chemical environment that includes various organic molecules (e.g., polysaccharides, proteins, amino acids, and lipids) as well as inorganic ions (e.g., calcium and phosphate at high levels). Although unimaginably rich in nutrients, this chemical environment is full of antimicrobial peptides, reactive oxidative species, hormones, cytokines, and neurotransmitters whose impact on the microbiota has yet to be comprehensively elucidated (50, 51). Resident microbes also constantly compete for niches with foreign microbial species that have traveled from the upper gastrointestinal tract. Furthermore, the aging of the host is usually accompanied by dampened lymphocyte function and increased risk of infection with pathogens such as *Clostridium difficile*; the result is more frequent occurrence of chronic inflammation and carcinogenesis in elderly hosts (52). In addition, intestinal diseases that are not related to the host's age (e.g., cancers and inflammatory bowel diseases) induce heightened cytokine production, uncontrolled inflammation, and tissue damage that also render the intestinal environment extremely hostile to the microbiota (53). Finally, oral administration of medications directly subjects the intestine and the intestinal microbiota to the effects of drugs like antibiotics and nonsteroidal anti-inflammatory agents. Therefore, the mammalian intestine selects for microbial colonizers that not only are metabolically adaptable but also possess efficient sensing and responding mechanisms for stress survival. How symbiotic intestinal bacteria manifest their stress response and persist in the

intestinal environment remains largely unknown but deserves extensive study.

As one of the most dominant Gram-negative bacterial genera in the intestine, *Bacteroides* must excel at dealing with stresses. Given its known functions in eukaryotic cells, sphingolipid-mediated signaling is an excellent candidate mechanism for the *Bacteroides* stress response. The highly flexible and dynamic nature of lipids guarantees timely transmission of signals. Situated at the membrane interface between cells and their external environments, sphingolipids are ideally positioned to broadcast information from outside the cell to the cytoplasm through lipid–lipid, protein–lipid, and protein–protein interactions. It is not surprising that genomic analyses of *Bacteroides* have revealed an unusually large number of extracytoplasmic function-type σ factors and hybrid two-component systems whose purposes are likely to be sensing and response (54). One possibility is that these membrane proteins are specifically and functionally associated with the sphingolipid-enriched domains. Downstream of these interactions is a profound transcriptional response. For example, *B. fragilis* can transcriptionally adjust 45% of its genome expression to withstand oxidative stress and become aerotolerant (55).

The data presented in this paper strongly suggest that *Bacteroides* organisms have the capacity to use sphingolipid-mediated mechanisms to respond to stress. This possibility raises several important questions. First, where are the sphingolipids located in bacterial membranes? The complicated envelope structures of *Bacteroides*, with double lipid membranes, lipopolysaccharides, extracellular polysaccharides, and capsular polysaccharides, certainly cloud the issue. The current model would predict that these lipids are likely enriched in the outer leaflet of the outer membrane, together with cholesterol molecules taken up from the intestinal environment. Second, what are the specific genetic components in *Bacteroides* sphingolipid signal transduction? Although tremendous progress has been made in understanding the protein constituents in eukaryotic sphingolipid signaling functional units, debate remains heated about a reliable protocol to separate "raft" domains and associated proteins from other membrane structures (56–58). Given the ability of *Bacteroides* to grow in a cholesterol-free environment where effective membrane organization and sphingolipid signaling are not possible, these bacteria present a unique opportunity to study this question. Cells grown under the cholesterol-free condition can be compared in terms of proteomic or transcriptional profiles with those grown in the presence of cholesterol and engaged in active signaling. This comparison allows one possible way to gain valuable information about important genes and proteins in signaling. Third, what are the signaling molecules? The enormous structural capacity and the functional specificity of sphingolipids make them ideal signaling molecules; yet the actual signals are known to be only transiently present at low concentrations and therefore difficult to analyze. Nevertheless, knowledge and methodology gained from studies of eukaryotic sphingolipids will be helpful in tackling this question in bacteria. Last, what is the biological significance of *Bacteroides* sphingolipid signaling? For example, does this mechanism mediate important functions in vivo to make *Bacteroides*

species successful as symbionts in mammalian hosts? Can similar mechanisms help other sphingolipid-containing symbiotic bacteria to survive (e.g., *Porphyromonas*, *Prevotella*)? What impact does this signaling have on microbial populations in the intestine that do not produce sphingolipids (e.g., Firmicutes)? Does this mechanism provide a common platform on which the host and the bacteria can share important information? Answers to these questions will help to better understand host–bacteria interactions.

Concluding Remarks. Past research has greatly advanced our appreciation of *Bacteroides* as symbionts; however, the survival of these bacteria in a challenging intestinal environment is poorly understood. We propose that *Bacteroides* species use a unique sphingolipid-mediated mechanism to establish a foothold in the enormously stressful intestinal environment, so that they are able to deliver important beneficial functions to the host. Further investigation of this proposal will likely reveal previously unknown metabolic and signaling pathways in *Bacteroides* and help to illuminate their relationship with the host.

Materials and Methods

Bacterial Stress Challenges. For challenges using rich-medium cultures, bacteria were grown to stationary phase in the presence or absence of 5 μM myriocin. Each sample was then pipette in an Eppendorf tube with 0.5 mL in volume and incubated for 14 h in a 42 °C water bath under aerobic condition. For challenges using minimal-medium cultures, bacteria were grown with 2 × 2 combinations of myriocin (5 μM; Sigma-Aldrich) and cholesterol (30 μg/mL; Sigma-Aldrich). Each sample, 0.5 mL in volume in an Eppendorf tube, was added with 1 μg/mL mitomycin C (Sigma-Aldrich) and followed by aerobic incubation for 2 h at 37 °C. Before and after treatments, CFUs/mL were counted. Each experiment was repeated at least three times.

Bacterial Lipid Purification and AFM Studies. Stationary-phase bacterial cells (50 mL) were centrifuged, and the cell pellets were resuspended in a mixture of methanol (20 mL) and chloroform (10 mL) in glass tubes. After the addition of 8 mL of water, each sample was extensively vortexed and centrifuged for phase separation. The lower organic phase was transferred to a new glass tube, and the water extraction step was repeated. Finally, the organic phase in a clean glass tube was dried with a gentle stream of N_2. Lipids were then weighed and resuspended in chloroform to a concentration of 0.5 mg/mL (59). For AFM studies, lipid samples were reconstituted into bilayers on a mica surface by a previously described vesicle fusion method (60, 61). In brief, the lipid sample was dried and resuspended in water by prolonged sonication. After all lipid molecules were dissolved, 20 μL of the solution was placed on a clean mica surface at 60 °C. The mica plate was then cooled down slowly to room temperature in a closed chamber so that the surface retained moisturized. The lipid bilayers were then examined by AFM (Veeco Multimode Nanoscope IIIA). The microscope was operated in the tapping mode according to standard procedure. Rastering of a probing tip (Olympus OMCL-AC160TS-W2) across a given area provided a height image that recorded the topography on top of the lipid bilayers. At least five images were taken for each sample.

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