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Excretion into feces of asialo GM1 in the murine digestive tract and *Lactobacillus johnsonii* exhibiting binding ability toward asialo GM1. A possible role of epithelial glycolipids in the discharge of intestinal bacteria

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Abstract In the digestive tract of mice (HR-1, 5 months old, ♀), asialo GM1 (GA1) exhibiting receptor activity toward several intestinal bacteria was preferentially expressed in the small intestine. Also, less than 10% of GA1 in the small intestine was converted into fucosylated and sulfated derivatives, but it was completely converted to fucosyl GA1 (FGA1) in the stomach, cecum and colon. Among the lipid components in these tissues, glycolipids other than Forssman antigen and cholesterol sulfate (CS) were present in the digestive tract contents. However, sulfated GA1, sulfatide and fucosyl GM1 in the gastrointestinal contents were not present in the cecal and colonic contents, in which the major glycolipids were ceramide monohexoside (CMH), GA1 and FGA1. The total amount of GA1 in the whole contents was 20% of that in the tissues. Thus, glycolipids were stable during the process of digestion, and excreted from the body together with cholesterol and CS. On the other hand, *Lactobacillus johnsonii* (LJ), whose receptor is GA1, was detected in the cecal and colonic contents on sequential analysis of 16S-ribosomal RNA and TLC-immunostaining of antigenic glycolipids with anti-LJ antiserum. LJ was found to comprise 20% of the total bacteria cultured in the lactobacillus medium under aerobic conditions, and to be present in the cecal and colonic contents, 9.8×10^7 cells versus 37 μg GA1 and 1.4×10^8 cells versus 49 μg GA1, respectively. Thus, GA1 in the contents might facilitate the discharge of intestinal bacteria by becoming attached them to prevent their irregular diffusion.

Keywords Sphingoglycolipids · Glyceroglycolipids · Molecular species · Lactobacillus · 16S-rRNA · TLC-immunostaining

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Abbreviations

The nomenclature for glycolipids is based on the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [1]

CMH	ceramide monohexoside
CS	cholesterol sulfate
18t:0	phytosphingosine
18d:1	sphingosine
24h:0	α-hydroxylignoceric acid
GA1	asialo GM1
FGA1	fucosyl GA1
FGM1	fucosyl GM1
SGA1	sulfated GA1
LJ	*Lactobacillus johnsonii*

Introduction

Asialo GM1 (GA1, Gg_4Cer) is known to constitute the receptor for several bacteria, *i.e. Lactobacillus casei, L. reuteri, L. johnsonii (L. acidophilus), Bifidobacterium*

bifidum, Pseudomonas aeruginosa, Actinomyces maeslundii and Neisseria gonorrhoeae, and to play an essential role in bacteria in the establishment of symbiosis or infection [2–4]. In the murine digestive tract, GA1 is abundant in the small intestine, but not in the stomach or colon, suggesting that the small intestine is the site of GA1-mediated formation of the bacterial flora for symbiosis [5]. However, although GA1 is a predominant glycolipid in the small intestine of germ-free mice, fucosyl GA1 (FGA1, $IV^2Fuc\alpha$-Gg_4Cer) is present in the small intestine of conventionally breeding mice [6]. The modification of GA1 to FGA1 has been revealed to occur on transcriptional induction of the α1,2-fucosyltransferase gene on infection by indigenous filamentous bacteria and wild type *Bacteroides thetaiotaomicron*, indicating that some bacteria regulate gene expression in the intestinal epithelial cells of the hosts, probably to suppress the growth of competitive bacteria [6–8]. Consequently, the flora formation through attachment to GA1 of symbiotic bacteria such as *Lactobacillus johnsonii* (LJ) is dynamically regulated through modification of GA1 by the coexisting bacteria [3, 4]. In addition, the expression of GA1 and FGA1 in the intestinal microvilli occurs as an event of the differentiation process during the migration of epithelial cells from the crypt to the top of villus [9], and the cells accompanying the bacterial flora are thought to be finally liberated into the digestive tract.

In the literature, comparison of glycolipids in the gastrointestinal tract and feces of germ-free and conventional rats demonstrated that feces contained intestinal glycolipids with ABO blood groups, and the amount of LacCer as the major glycolipid in the feces of conventional rats were higher than in germ-free mice [10]. Similarly, human intestinal glycolipids have been shown to be excreted into the feces, in which GlcCer, GM3 and Le^a, and GlcCer and LacCer were the major ones before and after the weaning period, respectively, and the reason why LacCer becomes the major glycolipid in human feces after the weaning period is the degradation by glycosidases produced by bacteria [11, 12]. However, since these studies were focused on structural characterization of glycolipids in feces, and not on quantitative analysis, the rates of excretion and degradation of epithelial glycolipids were unknown. Also, no detailed information on glycolipids in the contents of different regions of the murine digestive tract in comparison to those in the tissues and murine food is available so far.

Accordingly, we determined the total amounts and concentrations of glycolipids in the tissues and contents of various regions of the murine digestive tract, in which glycolipids were expressed in region-specific manners [5]. The information was thought to be useful for estimating not only the rate of turnover of epithelial glycolipids, but also the amounts of receptor glycolipids excreted together with bacterial flora. In this connection, we also determined the amounts of *Lactobacillus johnsonii*, whose receptor glycolipid is GA1 [2, 3], in the cecal and colonic contents through direct detection of bacterial glycolipids in lipid extracts of the contents by means of a newly developed method of TLC-immunostaining involving anti-LJ antiserum.

Materials and methods

Bacteria

The bacteria used in this study were purchased from the Japan Collection of Microorganisms (JCM, RIKEN, Wako, Saitama, Japan), and were as follows: *Lactobacillus johnsonii* (LJ) (JCM 1022) and *L. casei* (JCM 1134). The culture media for bacteria were as follows: MRS broth (Gibco-Invitrogen) and GYP (1 g glucose, 1 g yeast extract, 0.5 g peptone, 0.2 g meat extract, 0.2 g sodium acetate, 20 mg $MgSO_4$, 1 mg $MnSO_4$, 1 mg $FeSO_4$, 1 mg NaCl and 50 mg Tween 80 in 100 ml water). The number of lactobacilli was determined from the colony formation on a $CaCO_3$-containing agar-GYP plate (0.5% $CaCO_3$ and 1.2% agar in GYP medium) [13].

Mice

Mice (HR-1, female, 5 months old of age) were kept under conventional breeding conditions with lighting from 6:00 to 18:00 at a room temperature of 24±1°C and a humidity level of 55±10% with food (MF, Oriental Yeast, Tokyo) and water *ad lib*. Animal treatment followed the animal care guidelines of Kinki University. After anesthesia with pentobarbital (Abbott, Osaka), the tissues and contents from eight mice were collected separately. The upper and lower halves of the tract between the duodenum and cecum were used as the jejunum and ileum, respectively, whose contents were collected by injecting water with a syringe. The solid contents in stomach, cecum and colon were collected with a spatula. Also, a part of the cecal contents corresponding to 0.01–1 μg, was suspended in phosphate-buffered saline (PBS) and cultured on $CaCO_3$-agar GYP plates. The individual colonies on the plates were each picked up with a toothpick and then cultured in 80 ml of GYP medium.

Materials

Standard glycolipids from various sources were purified in our laboratory: GlcCer, LacCer, Gb_3Cer, Gb_4Cer and GM3 from human erythrocytes, Forssman glycolipid from equine kidney, and fucosyl GM1 (FGM1) from bovine thyroid. GA1 and FGA1 were prepared from GM1 and FGM1, respectively, by treatment with *Arthrobacter ureafaciens*

sialidase [14, 15]. *N*-Stearoyl glycolipids, as standards for TLC-densitometry, were prepared by deacylation with sphingolipid ceramide N-deacylase (Pseudomonas sp., Tk4), followed by reacylation with stearoyl chloride. Polyclonal antibodies to LJ, GA1, GM1 and Forssman glycolipids, and monoclonal antibodies to FGA1 (LFA-1), FGM1 (LFM-1), and sulfatides (TCS-1) were generated in our laboratory by immunizing rabbits or mice with LJ or purified glycolipids together with *Mycobacterium tuberculosis* or *Salmonella minnesota* as the adjuvant [5].

Analysis of lipids

Total lipids were extracted from the lyophilized tissues and contents with chloroform/methanol/water (20:10:1, 10:20:1 and 1:1:0, by vol.), and the combined extracts were used as the total lipids. A part of each extract was applied to a DEAE-Sephadex column (A-25, acetate form; GE Healthcare Bioscience, Piscataway, NJ, USA), and the unabsorbed and absorbed fractions were desalted by dialysis, being used as neutral and acidic lipids, respectively. Also, the neutral glycolipids were separated from the neutral lipids by acetylation, separation of the acetylated derivatives, deacetylation and desalting, whereas the acidic glycolipids were prepared from the acidic lipids by saponification with 0.5 M NaOH in methanol [16].

The total, acidic and neutral lipids, and acidic and neutral glycolipids thus obtained were applied on plastic-coated (Macherey-Nagel, Düren, Germany) and glass-coated (Merck, Darmstadt, Germany) TLC plates, which were then developed with n-hexane/diethylether/acetic acid (80:30:4, by vol.) for neutral lipids, chloroform/methanol/water (65:25:4, by vol.) for bacterial glycolipids and (65:35:8, by vol.) for neutral glycosphingolipids, chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, by vol.) for acidic glycolipids, and chloroform/methanol/acetone/acetic acid/water (8:2:4:2:1, by vol.) for cholesterol sulfate (CS) and sulfated glycolipids, and the spots were visualized with cupric acetate-phosphoric acid for neutral lipids, phospholipids and CS, Dittmer's reagent for phospholipids, and orcinol-sulfuric acid reagent for glycolipids. The densities of spots on TLC plates were determined by image analysis (NIH image). Standard lipids (0.1 to 1.5 μg), *i.e. N*-stearoyl derivatives of GlcCer, LacCer, Gb_3Cer, Gb_4Cer, Forssman glycolipid and GA1, FGM1, FGA1, triolein, oleic acid, cholesterol, sodium taurocholate and CS, were developed on the same TLC plates for the preparation of standard curves [5, 16].

Structural analysis of glycolipids

The individual glycolipids were purified using a silica gel (Iatrobeads 6RS8060; Iatron Laboratory, Tokyo) column by gradient elution with chloroform/isopropyl alcohol/water (85:15:0.2 and 40:60:2, by vol.) The purified ceramide monohexosides (CMH), diacylglyceryl dihexosides (DH-DG), GA1 and sulfated GA1 (SGA1, IV^3SO_3-Gg_4Cer) were analyzed by negative ion FABMS (JMS-700 TKM; JEOL, Tokyo) with triethanolamine as the matrix solvent. For determination of the compositions of fatty acids and carbohydrates, they were methanolized with 5% HCl in methanol at 80°C for 16 h. The resulting fatty acid methyl esters were extracted with n-hexane, and 1-*O*-methyl hexoses in the methanol phase were converted to trimethylsilyl derivatives with pyridine/hexamethyl disilazane/trimethylchlorosilane (10:2:1, by vol.) at 60°C for 5 min, followed by analysis with a GLC-mass spectrometer equipped with a DB-1 column (0.25 mmø×30 m) (Shimadzu, Kyoto). For characterization of SGA1, it was solvolyzed with 9 mM sulfuric acid in dimethyl sulfoxide/methanol (9:1, by vol.) at 80°C for 1 h, and the product was detected by TLC-immunostaining with anti-GA1 antiserum. Also, linkage analysis of carbohydrates was carried out by conversion of glycolipids to partially methylated aldohexitol acetates and *N*-methyl acetoamido aldohexitol acetates, followed by analysis with a GLC-mass spectrometer [16].

TLC-immunostaining

Lipids were developed on plastic-coated TLC plates (Macherey-Nagel), which were then blocked with blocking buffer (PBS containing 1% polyvinylpyrrolidone and 1% ovalbumin), and the spots were visualized by immunostaining with the above anti-LJ antibodies (1:500) diluted with dilution buffer (PBS containing 3% polyvinylpyrrolidone), followed by immunostaining with peroxidase-conjugated anti-rabbit IgG and IgM (1:1000; Jackson Immunoresearch Lab., PA, USA), and peroxidase substrates, 4-chloro-1-naphthol and H_2O_2, according to the procedure reported previously [5, 16].

Sequence of bacterial 16S-rRNA

Bacteria picked up with a toothpick were treated with proteinase K (10 mg/μl), EDTA (5 mM), NaCl (400 mM), and SDS (0.3%) in 20 mM Tris-HCl buffer (pH 8.0) at 55°C for 2 h, and the resultant DNAs were used for PCR with DNA polymerase (NovaTaq™ Hot Start DNA polymerase; Novagen, San Diego, CA, USA) in Ampdirect Plus (Shimadzu, Kyoto) under the following conditions: 27f sense primer, AGAGTTTGATCCTGGCTCAG, 1544r antisense primer, AGAAAGGAGGTGATCCAGCC, 95°C, 10 min, followed by 35 cycles of 94°C for 30 s, 53°C for 1 min and 72°C for 1 min, and then 72°C for 7 min [17]. The PCR products were purified with a MinElute PCR Purification Kit (Qiagen, MD, USA), and their sequences

were determined with a DNA analysis system with BigDye® Terminator Cycle Sequencing Reagent (v. 3.1) according to the manufacturer's instructions (ABI Prism 310 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA).

Results

Region-specific distribution of glycolipids in the murine digestive tract

In accord with our previous report [5], the distribution of glycolipids in the murine digestive tract was found to be region-specific, *i.e.* GA1 in the duodenum, jejunum and ileum, FGM1 in the stomach, and sulfatides in the stomach and cecum (Fig. 1). In addition to these major glycolipids, SGA1 was detected in the duodenum, jejunum and ileum (Fig. 1b and c). The structure of SGA1 was elucidated from the following evidence: positive reaction with anti-sulfatide antibodies (Fig. 1c), positive reaction with anti-GA1 antibodies of the products after solvolysis (Fig. 2), presence of 3-*O*-linked galactose detected on permethylation analysis, and the deprotonated molecular ions of SGA1, and the fragment ions of $[Gg_3Cer\text{-}1]^-$, $[LacCer\text{-}1]^-$, $[GlcCer\text{-}1]^-$, and $[ceramide\text{-}1]^-$ [18]. The extents of sulfation and fucosylation of GA1 were 7.6% and 8.1% in the duodenum, 6.0% and 0.3% in the jejunum, and 4.3% and 9.9% in the ileum, respectively, indicating that more than 85% of GA1 is expressed in these regions. Whereas, in the stomach, cecum and colon, FGA1, but not GA1 or SGA1, was detected, indicating complete fucosylation of GA1 in these regions (Fig. 3). The total amounts and concentrations of epithelial glycolipids and CS in several regions of the murine digestive tract are presented in Table 1. GA1 in the entire small intestine amounted to 674 μg, which was the highest among the glycolipids in these regions. In contrast to the region-specific distributions of GA1, FGA1, SGA1, FGM1 and sulfatide, CMH, Gb_4Cer, Forssman antigen, CS and GM3 were ubiquitously distributed in the digestive tract (Figs. 1 and 3). The tissues containing lipids in the highest amounts were as follows, , jejunum and ileum (CMH, GA1, SGA1 and CS), cecum (FGA1 and sulfatide), and stomach (FGM1) (Table 1).

Glycolipids in the contents of several regions of the murine digestive tract

The gastric contents of eight mice were less than 40% of that of a full stomach, passed through in about 5 h after ingestion, and their dry weights accounted for 6% of the total dried material in the entire digestive tract. The majority of the dried material in the tract were in the cecal and colonic contents, amounting to 35% and 46% of the total, respectively (Table 2). As shown in Fig. 4, triglycerides (TG), free fatty acids (FFA), phosphatidyl ethanolamine (PE), and phosphatidyl choline (PC), all of which were present in the murine food, greatly decreased from the gastric to the ileal contents, and they were almost completely absent in the cecal and colonic contents. In contrast, CMH, which was detected in the murine food and tissues was present in the contents of all regions of the digestive tract, and its relative concentrations in the cecal and colonic contents were higher than those in the gastric, jejunal and ileal contents (Fig. 4d and Table 2). On comparison of the molecular species of CMH in the cecal contents with those in the gastric and jejunal tissues by negative ion FABMS, CMH in the contents was found to be derived from both the gastro-intestinal epithelium and murine food. As shown in Fig. 5, the ceramide moiety of

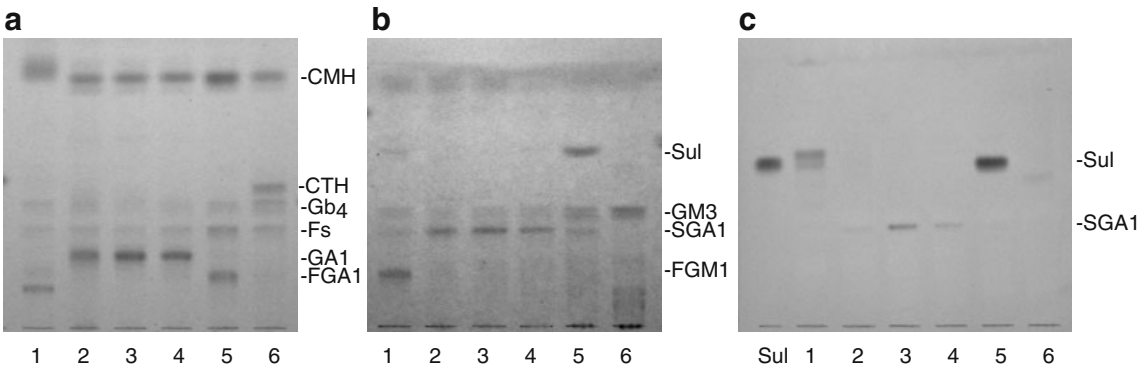

Fig. 1 TLC and TLC-immunostaining of glycolipids from tissues of the murine digestive tract. Neutral (**a**) and acidic (**b** and **c**) glycolipids, corresponding to 0.5 mg dry weight, were developed on plates with chloroform/methanol /water (65:35:8, by vol.) for A, and chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, by vol.) for B and C, and were detected with orcinol-sulfuric acid reagent for A and B, and with monoclonal anti-sulfatide antibody TCS-1 for C. 1, stomach; 2, duodenum; 3, jejunum; 4, ileum; 5, cecum; 6, colon; Sul, sulfatides; SGA1, IV^3SO_3-Gg_4Cer; CTH, ceramide trihexoside

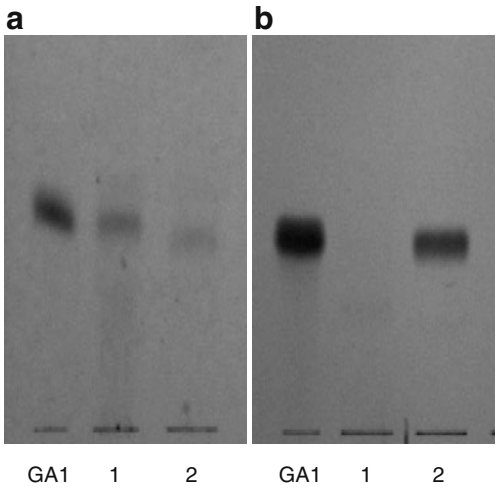

Fig. 2 TLC and TLC-immunostaining of SGA1 from the jejunum. SGA1 isolated from the jejunum (1) and its solvolyzed products (2) were developed with chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, by vol.), and were detected with orcinol-sulfuric acid reagent (**a**) and by TLC-immunostaining with anti-GA1 antiserum (**b**)

jejunal CMH preferentially comprised of α-hydroxy fatty acyl phytosphingosine: m/z 844, 18t:0–24h:0; m/z 830, 18t:0–23h:0; m/z 816, 18t:0–22h:0; m/z 788, 18t:0–20h:0; and m/z 732, 18t:–16h:0 (Fig. 5b). That of gastric CMH composed of α-hydroxy fatty acyl sphingosine and α-hydroxy fatty acyl phytosphingosine: m/z 872, 18t:0–26h:0; m/z 858, 18t:0–25h:0; m/z 844, 18t:0–24h:0; m/z 826, 18d:1–24h:0; m/z 812, 18d:1–23h:0; m/z 798, 18d:1–22h:0; m/z 770, 18d:1–20h:0; and m/z 714, 18d–16h:0 (Fig. 5a). And that of CMH in the cecal contents composed of a mixture of the above molecular species of gastro-intestinal CMH and CMH in the murine food, which were constituted of nonhydroxy fatty acyl phytosphingosine; m/z 828, 18t:0–24:0; m/z 800, 18t:0–22:0; and m/z 772, 18t:0–18:0 (Fig. 5c). Glucose was a major carbohydrate of CMH in the murine food, as well as that in the tissues and contents, and about 70% and 30% of CMH in the cecal contents was derived from the gastro-intestinal epithelium and murine food, respectively, as estimated from the relative intensities of molecular ions in Fig. 5c. These findings indicate that CMH is stable during the process of digestion and is excreted from the body. Similarly, the other epithelial glycolipids present in the contents were as follows, GA1 and FGA1 in the contents of all regions of the digestive tract, and sulfatides, FGM1 and SGA1 in the contents of the stomach, jejunum and ileum, but not those of the cecum and colon, indicating that the sulfuric and sialic acid groups of sulfatides, FGM1 and SGA1 are removed in the stomach, jejunum and ileum to yield CMH, FGA1 and GA1 in the cecal and colonic contents (Figs. 4d, e, and 6). In the jejunal and ileal contents, GA1 was the major glycolipid, comprising about 60% of the total glycolipids, whose concentration was similar with those in the jejunal and ileal tissues. Also, GA1 and FGA1 in the cecal and colonic contents were present in concentrations of 0.57 μg and 0.02 μg per mg dry weights, and total amounts of 86.2 μg and 3.0 μg, respectively (Table 2). For comparison, the total amounts of several lipids in the contents from different regions of the digestive tract are shown in Fig. 7. PC and free fatty acids from both the murine food and epithelial tissues greatly decreased from the gastric to the ileal contents on enzymatic hydrolysis and incorporation of free fatty acids into the tissues, respectively. Bile acids, which were secreted from the gallbladder and played an essential role in the process of lipid digestion, were the major lipids in the jejunal and ileal contents and, to a lesser extent, in the gastric contents, and were absent in the cecal and colonic contents. Consequently, the major lipids accumulated in the cecal and colonic contents to be excreted from the body were cholesterol, CS, GlcCer, GA1 and FGA1. The total amounts of GA1 and FGA1 in the cecal and colonic contents corresponded to 12.8% and 2.5% of those in the epithelial tissues of the entire digestive tract (Table 1 and Fig. 7).

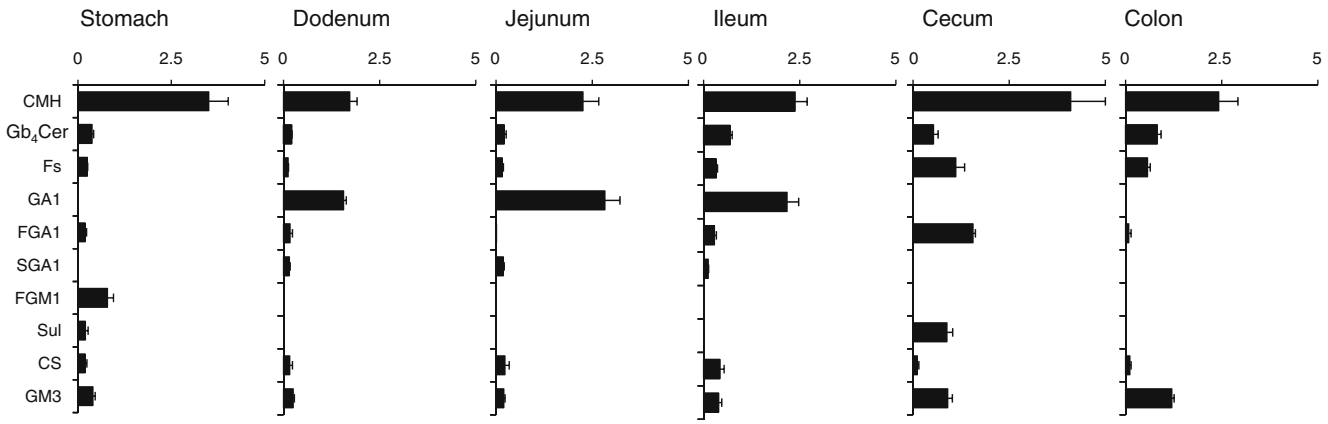

Fig. 3 Amounts of glycolipids and CS in tissues of the murine digestive tract (μg/mg dry tissue weight)

Table 1 Total amounts of epithelial glycolipids in the digestive tract of mice (HR-1, ♀, 5 months old)

		Stomach	Duodenum	Jejunum	Ileum	Cecum	Colon
Dry tissue weight (mg/mouse)		46.5±3.5	22.8±2.8	134.0±19.3	119.5±6.1	25.8±7.2	45.2±1.9
CMH	(μg/mg)	3.5±0.5	1.7±0.2	2.3±0.4	2.4±0.3	4.1±0.9	2.4±0.5
	(μg/mouse)	164.1±24.2	39.2±4.3	302.8±54.9	284.4±37.0	105.8±23.2	109.4±22.6
GA1	(μg/mg)	–	1.6±0.1	2.8±0.4	2.2±0.3	–	–
	(μg/mouse)	–	35.6±1.6	379.2±52.3	259.3±35.9	–	–
FGA1	(μg/mg)	0.19±0.04	0.17±0.06	0.01±0.00	0.28±0.05	1.56±0.06	0.08±0.06
	(μg/mouse)	8.7±1.9	3.9±1.4	1.3±1.3	33.5±6.0	40.2±1.5	3.6±2.7
SGA1	(μg/mg)	–	0.15±0.02	0.19±0.02	0.12±0.01	tr	
	(μg/mouse)	–	3.4±0.5	25.5±2.7	14.3±1.2	–	–
FGM1	(μg/mg)	0.79±0.16	–	–	–	–	–
	(μg/mouse)	36.7±7.4	–	–	–	–	
Sulfatide	(μg/mg)	0.20±0.07	–	–	–	0.88±0.15	–
	(μg/mouse)	9.3±3.3	–	–	–	22.7±3.9	–
CS	(μg/mg)	0.19±0.05	0.16±0.07	0.23±0.07	0.42±0.11	0.11±0.04	0.11±0.03
	(μg/mouse)	8.8±2.3	3.6±1.6	30.8±9.4	50.2±13.1	2.8±1.0	5.0±1.4

Values are the means of eight samples

Lactobacillus johnsonii in the cecal and colonic contents

Rabbit polyclonal anti-*Lactobacillus johnsonii* (LJ) antisera reacted with di-, tri- and tetrahexaosyl diglycerides as the major antigens in the cell wall of LJ, and also reacted with di- and trihexaosyl diglycerides in *Lactobacillus casei* (LC), but the glycolipid profiles detected on TLC-immunostaining of LJ were clearly distinct from those of LC (Fig. 8). The structures of dihexaosyl diglycerides (DH-DG) in LJ and LC were identical with each other and were determined to be as Galα1-2Glcα-DG by permethylation analysis, and enzyme treatment with α-galactosidase and α-glucosidase, as reported in the literature [19, 20]. The amounts of glycolipids in LJ were estimated to be 0.20 μg DH-DG, 0.17 μg TriH-DG and 0.22 μg TetH-DG per 1×10^8 cells, respectively. On cultivation of the cecal contents in MRS broth, followed by analysis of the bacterial glycolipids by TLC-immunostaining, the same glycolipids as those in LJ were detected in the lipid extracts of bacteria cultured under both aerobic and anaerobic conditions (lines A and N in Fig. 8). Also, when fresh cecal contents (0.01–1 μg wet weight) were cultured on a $CaCO_3$-agar GYP

Table 2 Amounts of glycolipids in contents of several regions of the digestive tract of mice (μg/mg dry wt)

	Murine Food	Gastric Contents	Jejunal Contents	Ileal Contents	Cecal Contents	Colonic Contents
Dry weight (mg/mouse)	–	12.0±1.2	7.5±1.3	16.1±4.0	65.4±1.0	85.8±0.7
Free fatty acid	0.9±0.2	16.6±0.2	8.9±0.2	1.9±0.1	0.05±0.01	0.08±0.02
Cholesterol	0.2±0.1	3.47±0.1	3.8±0.4	2.9±0.2	1.3±0.4	0.9±0.5
CS	–	0.4±0.1	0.6±0.1	0.7±0.1	0.2±0.1	0.4±0.1
Sulfatide	–	0.18±0.02	0.07±0.01	0.05±0.01	–	–
Bile acids	–	1.3±0.4	7.8±1.2	5.9±1.5	–	–
PC/PS	0.4±0.03	6.1±1.4	7.8±1.8	3.3±0.3	–	–
LPC	tr	tr	0.2±0.03	0.07±0.01	–	–
CMH	0.05±0.04	0.82±0.11	0.64±0.08	0.80±0.05	0.97±0.06	1.13±0.09
GA1	–	0.42±0.06	1.17±0.13	2.11±0.61	0.57±0.07	0.57±0.02
FGA1	–	0.05±0.02	0.04±0.01	0.06±0.02	0.02±0.01	0.02±0.01
SGA1	–	0.08±0.02	0.21±0.05	0.48±0.05	–	–
FGM1	–	0.06±0.02	0.02±0.01	0.02±0.01	–	–

Values are the means of eight samples

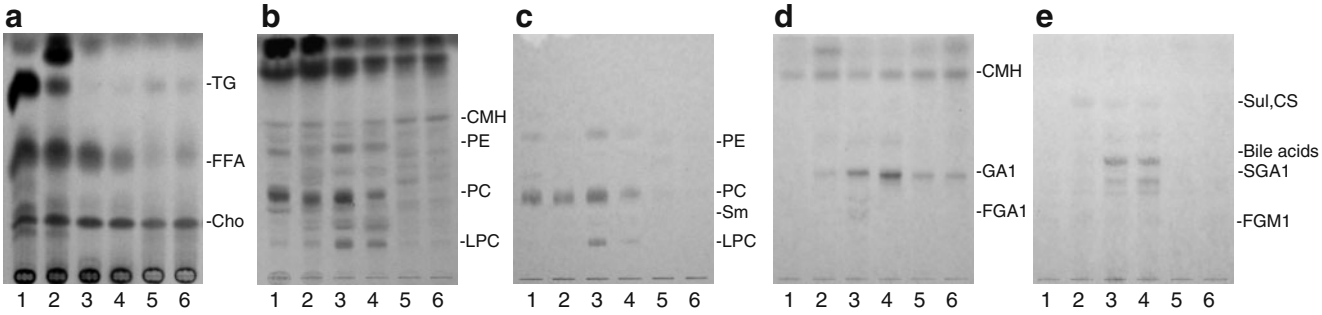

Fig. 4 TLC of lipids in the murine food and the contents of several regions of the murine digestive tract. Total lipids (**a**, **b** and **c**), and neutral (**d**) and acidic glycolipids (**e**) were developed with n-hexane/diethyl ether/acetic acid (80:30:4, by vol.) for **a**, chloroform/methanol/water (65:35:8, by vol.) for **b** and **c**, and chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, by vol.) for **d** and **e**, and were detected with cupric acetate-phosphoric acid for **a** and **b**, Dittmer's reagent for **c**, and orcinol-sulfuric acid reagent for **d** and **e**. 1, murine food; 2, stomach contents; 3, jejunal contents; 4, ileal contents; 5, cecal contents; 6, colonic contents. The lipids from the murine food, corresponding to 2 mg dry weight for **a**, **b** and **c**, and 5 mg dry weight for **d** and **e**, and those from the contents, corresponding to 0.2 mg dry weight for **a**, **b** and **c**, and 0.5 mg dry weight for **d** and **e**, were applied on a TLC-plate, respectively

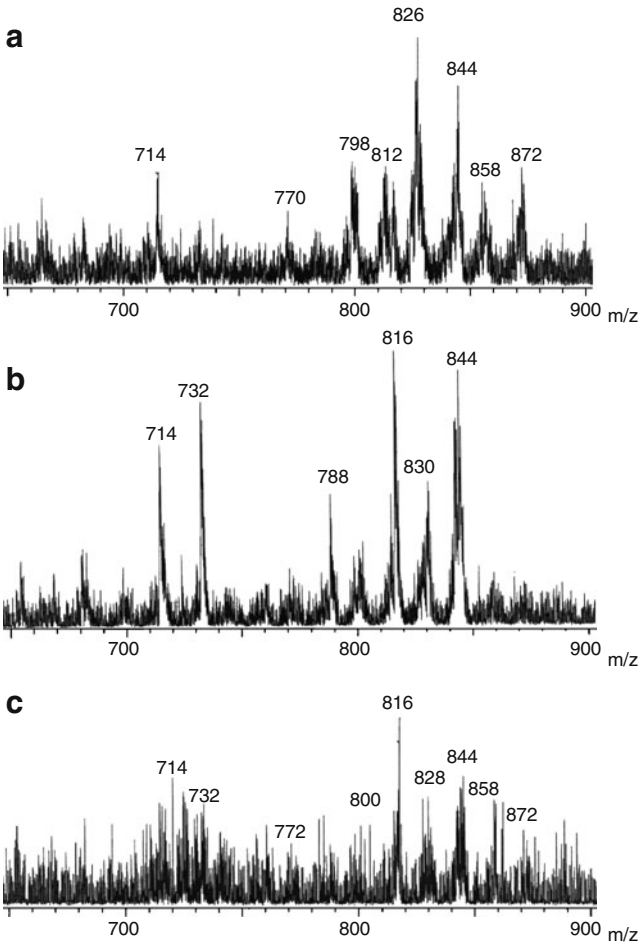

Fig. 5 Negative ion FABMS spectra of CMH from gastric tissues (**a**), jejunal tissues (**b**), and cecal contents (**c**). Triethanolamine was used as the matrix solution

plate under aerobic conditions, colonies with a clear zone, indicating the production of lactic acid, were obtained, (4.3–7.8) x 10^2 cells per μg wet weight of the contents. All colonies, less than 30 obtained at the higher dilution, were picked up with a toothpick, and their 16S-rRNA and glycolipid antigens were determined by DNA sequencing and TLC-immunostaining, respectively. As shown in Fig. 8, colonies 7 and 8 exhibited identical DNA sequences and identical glycolipid profiles to those of LJ, indicating that these colonies were certainly LJ. LJ comprised about 20% of the total colonies, the other colonies being of *Enterococcus faecalis*, and the mean number of LJ measured by colony formation was determined to be 1.1×10^6 cells per mg dry weight of the cecal contents. Then, we attempted to determine the number of LJ by direct detection of glycolipid antigens in lipid extracts of the cecal and colonic

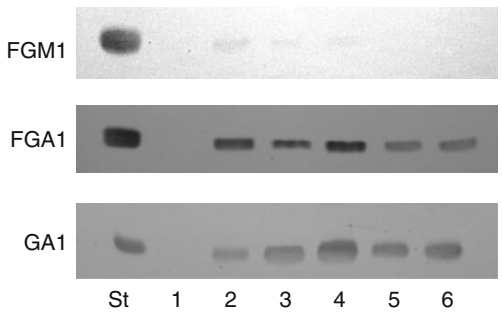

Fig. 6 TLC-immunostaining of glycolipids in the murine food and contents of several regions of the murine digestive tract. Neutral (FGA1 and GA1) and acidic(FGM1) glycolipids, corresponding to 5 mg dry weight of the murine food and 0.5 mg dry weight of the contents, were developed on plastic-coated TLC plates with chloroform/methanol /0.5% $CaCl_2$ in water (65:45:10, by vol.), and were detected with anti-FGM1, anti-FGA1 and anti-GA1 antibodies. 1, murine food; 2, stomach contents; 3, jejunal contents; 4, ileal contents; 5, cecal contents; 6, colonic contents

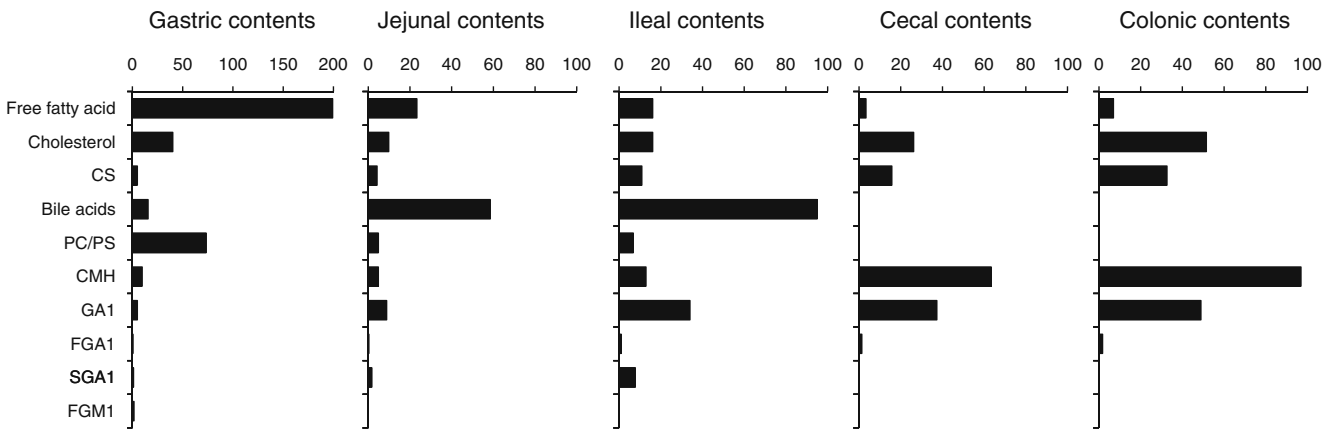

Fig. 7 Total amounts of lipids in the contents of several regions of the murine digestive tract (μg/mouse)

contents by TLC-immunostaining with anti-LJ antiserum (Fig. 9). With this procedure, the cell number as a function of the amount of glycolipids was linear from 1×10^6 to 50×10^6 cells of LJ, the limit of detection being 1×10^6 cells. On TLC-immunostaining, the cecal and colonic contents were revealed to contain 1.5×10^6 and 1.6×10^6 cells of LJ per mg dry weight, corresponding to 9.8×10^7 and 1.4×10^8 cells of LJ in the total cecal and colonic contents, respectively, which were discharged from the body together with 37 μg and 49 μg of its receptor, GA1, respectively (Fig. 7).

Discussion

As reported in this paper, sulfoglycolipids and ganglioseries glycolipids in the tissues of the murine digestive tract were found to be expressed in a region-specific manner. GA1, as the receptor, for several bacteria including lactobacilli, was preferentially expressed in the duodenum, jejunum and ileum, and its total amounts were the highest among glycolipids in these regions, amounting to 674 μg in the entire small intestine. In accord with its high amount in the small intestine, GA1 was the predominant glycolipid in the jejunal and ileal contents, comprising about 60% of the total glycolipids, probably due to the release of epithelial cells from the microvilli into the tract. Also, the relative ratios of SGA1 to GA1 in contents (14–17%) were higher than those in the tissues (4–8%), suggesting that cells containing SGA1 in relatively high amounts are readily released into the tract to give the high ratios in the contents. SGA1, GA1 and bile acids in the gastric contents were thought to be derived from the small intestine through backward flow. Similarly, since sulfatide and CS were detected in the gastro-intestinal contents at concentrations equivalent to those in the respective tissues, cells with sulfated lipids were thought to be released, to a great extent, into the tract. In contrast, Gb_4Cer and Forssman antigen in the stromal region of the villi were not detected in the contents, even in trace amounts [21].

On comparison of the lipid compositions in the contents of different regions of the digestive tract, the sites of degradation and absorption of individual lipids were clearly

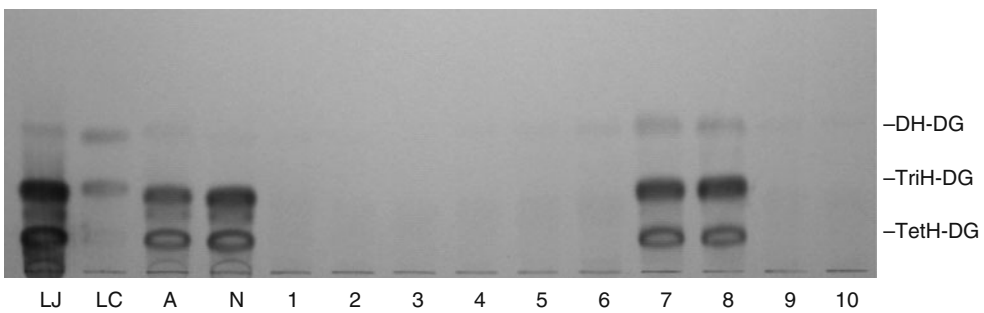

Fig. 8 TLC-immunostaining with anti-LJ antiserum of lipids from bacteria in murine cecal contents. Cecal contents (about 0.1 μg) were directly cultured in 30 ml of GYP medium under aerobic (*A*) and anaerobic (*N*) conditions at 37°C overnight, and were also cultured on $CaCO_3$-agar GYP plates under aerobic conditions, and individual colonies were cultured in 30 ml of GYP medium under aerobic conditions. The bacteria thus obtained were lyophilized, and then their lipids, corresponding to 1 mg dry weight, were developed on TLC plates with chloroform/methanol /water (65:25:4, by vol.), and were detected by TLC-immunostaining with anti-*LJ* antiserum. *LJ*, lipids from *LJ*; *LC*, lipids from *L.casei*; 1–10, lipids from bacteria in individual colonies on a $CaCO_3$-agar GYP plate

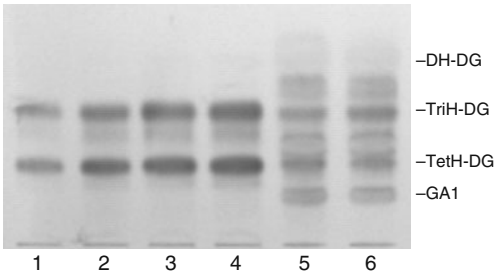

Fig. 9 TLC-immunostaining with anti-LJ antiserum of lipids from cecal and colonic contents. Lipids from LJ (1, 4.2×10^6; 2, 8.4×10^6; 3, 16.8×10^6; 4, 25.2×10^6), and the cecal (*5*) and colonic (*6*) contents, corresponding to 4 mg dry weight, were analyzed by TLC-immunostaining with anti-LJ antiserum, which also reacted with GA1 in the contents, as reported previously [5]

observed to be as follows, TG in the stomach, phospholipids in the jejunum, and free fatty acids in the ileum. The major esterified lipids in the murine food and derived from the epithelial cells, *i.e.* TG and phospholipids, and their degradation products, lysophospholipids and free fatty acids, respectively, were absent in the region between the ileum and the cecum, together with bile acids. Cholesterol, CS, GlcCer, GA1 and FGA1 became the major lipids in the cecal and colonic contents, whose lipid compositions were identical with those in feces. Since sulfatides, SGA1 and FGM1 present in the gastro-intestinal contents were not detected in the cecal and colonic contents, sulfatases and sialidases that cleave these glycolipids were present, but enzymes that cleave the glycosidic linkages in GlcCer, GA1 and FGA1 were supposed to be absent, although neutral ceramidase was reported to be present in the small intestine [22]. On the other hand, because CS sulfatase is generally insoluble in nature and is supposed not to be secreted into the digestive tract, CS was thought to be accumulated in the cecal and colonic contents [23].

As determined from the relative ion intensities of the molecular ions observed on a negative ion FABMS, about 30% of GlcCer in the cecal and colonic contents, was derived from the murine food, in which GlcCer was present at the concentration of 0.05 μg/mg dry weight, whereas GA1 and FGA1 in the cecal and colonic contents preferentially originated from the epithelial cells in the digestive tract. The total amount of GA1 in the cecal and colonic contents, a part of which was derived from SGA1, was 86 μg, corresponding to about 12% of the total GA1 in the tissues, indicating that a large amount of GA1 is excreted from the body. Since GA1 is responsible for the receptor activity toward bacteria, a possible role of GA1 in the contents was thought to be related with the discharge of bacterial flora attached to GA1. Namely, the bacterial flora attached to GA1 on intestinal epithelial cells migrates from the crypt to the villus of microvilli, and is finally liberated into the digestive tract. Even after degradation of epithelial cells during the digestion process, GA1 in the contents was supposed to remain bound to prevent the irregular spreading of bacteria in the tract. Practically, we attempted to detect LJ exhibiting binding activity toward GA1 in the cecal and colonic contents by colony formation, followed by characterization of individual colonies by sequential analysis of 16S-rRNA and detection of antigenic glycolipids with anti-LJ antisera. About 20% of the colonies obtained on cultivation of the cecal contents under aerobic conditions were shown to be LJ, amounting to 1.1×10^6 cells per mg dry weight. In addition, LJ in the cecal and colonic contents was estimated to comprise 1.5×10^6 and 1.6×10^6 cells per mg dry weight, respectively, by direct detection of antigenic glycolipids in lipid extracts with anti-LJ antiserum. The colony formation essentially indicated the living cells, whereas TLC-immunostaining indicated living, resting and dead cells, causing the difference in the cell numbers obtained with the two procedures. However, as clearly shown in Fig. 8, TLC-immunostaining with anti-LJ antiserum made it possible to detect LJ in lipid extracts of the cecal and colonic contents, indicating that it is applicable to the quantitative detection of LJ in materials stored after lyophilization, as well as fresh ones. As calculated from the average molecular weight of GA1 containing *N*-cerebronoyl phytosphingosine (1372), 37 μg of GA1 in the cecal contents represented 27 nmole, corresponding to 1.6×10^{16} molecules, which was sufficient for one-to-one binding of 9.8×10^7 cells of LJ in the cecal contents. Although anti-LJ antiserum reacted with Galα1-2Glcα1-3DG, as a common glycolipid of lactobacillus species, in our preliminary experiment, TriH-DG and TetH-DG were not detected in the ratios observed in LJ in other lactobacilli, *i.e. L. casei, L. fermentum, L. plantarum, L. ruteri* and *L. rhamnosus,* indicating that both glycolipids exhibit strong antigenicity and that anti-LJ antiserum is a useful tool for detection of symbiotic lactobacilli, *e.g.* LJ. Similarly, glycolipids characteristic of individual bacterial species can be probably detected by direct immunization with the respective bacteria.

In this connection, the strong antigenicities of DG-type glycolipids in Gram-positive bacteria and lipooligosaccharides in Gram-negative bacteria have been well recognized to be useful for the serotyping of bacterial species [13, 24], and to cause the production of natural antibodies related to blood group antigens in normal human sera [25], and of disease-related antibodies in patients suffering from multiple sclerosis [26] and Guillan-Barré syndrome [27, 28]. In addition to DG-type glycolipids, structures mimicking the oligosaccharide of GA1 were found to be present in glycoprotein of LJ, and to generate antibodies against GA1 on immunization with LJ, as shown in Fig. 9. The resemblance of the epitope structures in bacteria and a host might be essentially related with the mechanism for evading immune responses and allow LJ symbiosis in the

digestive tract, as reported previously [5]. Thus, the immune system of a host animal recognizes individual bacteria through glycolipids in the bacterial cell wall, and bacteria utilize these glycolipids in the host as receptors, indicating that cross-talk mediated by glycolipids between bacteria and the host is essential for the establishment of symbiosis.

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