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Bacterial species-characteristic profiles of molecular species, and the antigenicity of phospholipids and glycolipids in symbiotic Lactobacillus, Staphylococcus and Streptococcus species

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Abstract Human symbiotic bacteria, *Lactobacillus reuteri* (LR) in the intestines, *Staphylococcus epidermidis* (SE) in skin and *Streptococcus salivalis* (SS) in the oral cavity, contain dihexaosyl diglycerides (DH-DG) in concentrations equivalent to those of phosphatidyl glycerol (PG) and cardiolipin (CL), together with mono- to tetrahexaosyl DGs. The molecular species, as the combination of fatty acids in the DG moiety, were revealed to be bacterial species-characteristic, but to be similar between glycolipids and phospholipids in individual bacteria, the major ones being 16:0 and cy19:0 for LR, ai15:0 and ai17:0 for SE, and 16:0 and 18:1 for SS, respectively. The carbohydrate structures of DH-DGs were also bacterial species-characteristic, being Galα1-2Glcα for LR, Glcβ1-6Glcβ for SE, and Glcα1-2Glcα for SS, respectively. Also, bacterial glycolipids were revealed to provide antigenic determinants characteristic of bacterial species on immunization of rabbits with the respective bacteria. Anti-*L. johnsonii* antiserum intensely reacted with tri- and tetrahexaosyl DGs, in which Galα was bound to DH-DG through an α1-6 linkage, as well as with DH-DG from LR. Although anti-SE antiserum preferentially reacted with DH-DG from SE, anti-SS antiserum reacted with DH-DG from SS and, to a lesser extent, with DH-DGs from LR and SE. But, both anti-SE and anti-SS antiserum did not react at all with monohexaosyl DG or glycosphingolipids with the same carbohydrates at the nonreducing terminals. In addition, 75 % of human sera, irrespective of the ABO blood group, were found to contain IgM to tri- and tetrahexaosyl DGs from LR, but not to DH-DGs from LR, SE and SS.

Keywords Symbiotic bacteria · Glycerophospholipids · Glyceroglycolipids · Sphingoglycolipids · Anti-bacterial antiserum · TLC-immunostaining

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Abbreviations: The nomenclature for glycosphingolipids is based on the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [1].

CL	cardiolipin
DG	diglycerides
MH-DG	monohexaosyl diglycerides
DH-DG	dihexaosyl diglycerides
PA	phosphatidic acid
PG	phosphatidyl glycerol
TH-DG	trihexaosyl diglycerides
TetH-DG	tetrahexaosyl diglycerides
cy	cyclopropane
ai	anteiso

LJ *Lactobacillus johnsonii*
LR *Lactobacillus reuteri*
SE *Staphylococcus epidermidis*
SS *Streptococcus salivalis*

Introduction

As reported previously [2], the major lipid constituents of Gram-positive bacteria belonging to the Lactobacillus species are glycerophospholipids and glyceroglycolipids, among which dihexaosyl diglycerides (DH-DG) are present in concentrations equivalent to those of phospholipids. However, the carbohydrate structure of DH-DG is known to be bacterial species-specific, and its modification occurs in individual strains of the same bacterial species. All Lactobacillus strains examined contained Galα1-2Glcα1-3′DG as a Lactobacillus species-characteristic glycolipid, and tri- and tetrahexaosyl DGs, in which α-Gal is linked at the nonreducing terminal of Lactobacilli-characteristic DH-DG through an α1-6 linkage, have been detected in the intestinal Lactobacilli of several animal species, *i.e. L.johnsonii* (LJ) and *L. intestinalis,* but not in non-intestinal *L. fermentum* and *L. plantarum* [2]. On immunization of rabbits with LJ, antibodies against Galα1-2Glcα1-3′DG, Galα1-6Galα1-2Glcα1-3′DG (TH-DG), and Galα1-6Galα1-6Galα1-2Glcα1-3′DG (TetH-DG), but not against phospholipids, have been readily yielded, indicating their strong antigenicities. Since anti-LJ antisera exhibit sufficiently high specificity and titers toward these glycolipids, particularly toward TH-DG and TetH-DG, they have been used for the sensitive detection and quantitation of bacteria belonging to the Lactobacillus species in intestinal contents [3, 4]. In addition, antibodies to them have been detected in normal human sera, indicating that human immune surveillance ability as to intestinal symbiotic Lactobacilli is partly gained through the recognition of bacterial glycolipids as target antigens [2]. To further characterize the antigenic properties of bacterial glycolipids in relation with symbiosis, antisera against *Staphylococcus epidermidis* (SE) in skin and *Streptococcus salivalis* (SS) in the oral cavity were generated by direct immunization of rabbits with them.

Also, DH-DGs in Lactobacillus and Staphylococcus species have been revealed to be composed of unique fatty acids, *i.e.* cyclopropane ring-containing cy19:0 fatty acid (11,12-methylene-octadecanoic acid) and anteiso-type (ai15:0 and ai17:0) fatty acids, respectively [2]. In general, the hydrophobic chains of lipids in mammalian tissues and cells are known to differ between glycolipids and phospholipids, and between phospholipids distributed in the inner and outer leaflets of the lipid bilayer [5–7]. In the case of glycosphingolipids and sphingomyelin, the fatty acids of the ceramide moieties are synthesized by ceramide synthases specific as to the chain length of acyl CoA [8] and even after their synthesis, selection of the ceramide structures in glycosphingolipids occurs during the process of elongation of carbohydrate chains, particularly for those of gangliosides, because of the increasing ratio of C20-sphingosine-containing to C18-sphingosine-containing ceramides in mono- to tetrasialosyl gangliosides [9]. Whereas in the synthetic pathway for glycerophospholipids, systematic deacylation and reacylation reactions start after the synthesis of phosphatidic acid (PA) from glycerol 3-phosphate and acyl CoA to give characteristic molecular species of individual phospholipids, which are important for the construction of biomembranes and phospholipid-mediated functions including signal transduction through biomembranes [10, 11]. Accordingly, mass spectrometric analysis of the molecular species of glycolipids and phospholipids in human symbiotic bacteria, *i.e. L. reuteri* (LR), SE and SS, has been carried out to clarify their metabolic relationships and structural characteristics in different habitats, *i.e.* the intestines, skin and oral cavity, respectively.

Materials and methods

Bacteria L. johnsonii (LJ, JCM 1022), *L. reuteri* (LR, JCM 1112), *Staphylococcus epidermidis* (SE, JCM 2414), and *Streptococcus salivalis* (SS, JCM 5707) were purchased from the Japan Collection of Microorganisms (JCM), RIKEN BioResource Center (Wako, Saitama, Japan). *S. aureus* (860) and *S. epidermidis* (866), and *E. coli* (JM109) were also obtained from the American Type Culture Collection (Rockville, MD, USA) and Invitrogen (Carlsbad, CA, USA), respectively. The culture media for bacteria were as follows: MRS broth (Beckton-Dickinson, Sparks, MD, USA) for Lactobacilli, tryptic soy broth (Beckton-Dickinson) for SE, heart infusion broth (Beckton-Dickinson) for SS, and LB broth (Nacalai Tesque, Kyoto) for *E. coli*.

Materials Glycolipids, GlcCer, GalCer, LacCer, Gb_3Cer, Gg_3Cer, Gb_4Cer, Gg_4Cer, Lc_4Cer, nLc_4Cer, $IV^3GalNAc\alpha$-Gb_4Cer and $IV^3Gal\alpha$-nLc_4Cer, were purified in our laboratory [3, 4]. Phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), cardiolipin (CL), and peroxidase-conjugated anti-rabbit IgG and anti-human IgM antibodies were purchased from Sigma (St. Louis, MO, USA). The concentrations of standard phospholipids in chloroform/methanol (1:1, by vol.) were determined by the phosphomolybdate procedure after decomposition of the lipids with 70 % $HClO_4$ and H_2O_2 [12]. Normal human sera, blood groups A, B, AB and O, were obtained from Keio University Hospital, and used according to the guidelines of the Committee for Informed Consent.

Antiserum Antisera toward LJ, SE and SS were generated by immunizing rabbits (Japanese white ♀, 3.5 kg; Japanese Biological Materials, Tokyo) intradermally with a water-in-oil emulsion prepared by mixing 15 mg of each bacterium in 1 ml of phosphate-buffered saline (PBS) with 1 ml of Freund's complete adjuvant (Sigma), on the initial day and 2 weeks after the initial injection, and the antibody titers of sera after 4 weeks were subsequently monitored by enzyme-linked immunosorbent assaying (ELISA) and bacterial aggregation with the respective bacterium as the antigen.

Quantitation of bacterial lipids The lipid extracts derived from bacteria were partitioned by Folch's procedure [13], and then aliquots corresponding to 0.5–1.0 mg dry weight were separated on glass-coated (Merck, Darmstadt, Germany) and plastic-coated (Macherey-Nagel, Düren, Germany) TLC plates with chloroform/methanol/water (65:25:4. by vol.). Detection was performed with cupric acetate-phosphoric acid reagent for all lipids, Dittmer's reagent for phospholipids, ninhydrin reagent for aminolipids and orcinol-sulfuric acid reagent for glycolipids, and by TLC-immunostaining with rabbit anti-LJ, anti-SE and anti-SS antisera (1:500), and human sera (1:100). Standard lipids, *i.e.* PG, CL, and N-stearoyl derivatives of GalCer, LacCer, Gb_3Cer, Gb_4Cer (0.1–1.5 μg), were developed on the same TLC plates and after visualization of phospholipids with cupric acetate-phosphoric acid and of glycolipids with orcinol-sulfuric acid, the density of spots was determined by image analysis (NIH image) for the preparation of standard curves.

Enzyme-linked immunosorbent assay (ELISA) Purified DH-DGs from LR, SE and SS, and TH-DG and TetH-DG from LR were dissolved in ethanol by sonication (0.5 μg /100 μl), and then put in individual wells of a microtiter plate, which was left at room temperature until the ethanol had completely evaporated off. The plate was blocked with 100 μl of bovine serum albumin (BSA, 1 %) in PBS at 4°C overnight, and then to the plate, 100 μl of human serum diluted 1:100 and 1:200 with 1 % BSA in PBS was added, followed by reaction at room temperature for 2 h. After washing the plate with 0.1 % Tween 20 in PBS five times, the antibodies bound on the plate were detected by reaction with 100 μl of peroxidase-conjugated anti-human IgM antibodies diluted 1:2000 with 1 % BSA in PBS at room temperature for 2 h, followed by reaction with o-phenylenediamine (4.6 mM) and H_2O_2 (0.015 %) in 25 mM citrate-phosphate buffer (pH 5.0) as the substrates for peroxidase at room temperature for 15 min. The reaction was terminated by the addition of 4 M sulfuric acid (20 μl), and then the optical density at 490 nm was determined [14]. The background values obtained on reaction with human sera in wells without glycolipids under the same conditions were subtracted from the values obtained above.

Purification of glycolipids and phospholipids Total lipids were extracted from the lyophilized bacteria with chloroform/methanol/water (20:10:1, 10:20:1 and 1:1:0, by vol.), and then fractionated into neutral and acidic lipid fractions by DEAE-Sephadex (A-25, acetate form; GE Healthcare Bioscience, Piscatway, NJ, USA) column chromatography [14]. Glycolipids, and CL and PG in LR and SE were clearly separated into the neutral and acidic fractions, respectively, but one of the glycolipids in SS was eluted in the acidic lipid fraction together with PG and CL, and two glycolipids in the neutral lipid fraction. After Folch's partitioning, individual glycolipids and phospholipids were purified from each fraction using a silica gel (Iatrobeads 6RS8060; Iatron Laboratory, Tokyo) column by gradient elution with chloroform/methanol/water (85:15:0.2, 70:30:4, and 10:90:4, by vol.). The purity of purified lipids was examined by TLC as described above.

Structural analysis of glycolipids and phospholipids The purified glycolipids and phospholipids were analyzed by positive and negative ion FAB-MS (JMS-700TKM; JEOL, Tokyo), respectively, with triethanolamine (TEA) as the matrix, and proton magnetic resonance spectroscopy (JNM-ECP700, JEOL) with dimethyl sulfoxide-d6/D_2O (98:2, by vol.). For determination of the fatty acid and carbohydrate compositions, 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 into trimethylsilyl derivatives with pyridine/hexamethyl disilazane/trimethylchlorosilane (10:2:1, by vol.) at 60°C for 5 min, followed by analysis with a GC-MS (GP5050: Shimadzu, Kyoto) equipped with a DB-1 column (0.25 mmø x 30 m) from 150°C to 250°C at 10°C/min. Also, linkage analysis of carbohydrates was carried out by conversion of glycolipids to partially methylated aldohexitol acetates, followed by analysis with a GC-MS with a DB-1 column from 150°C to 210°C at 4°C/min [2–4]. In addition, for determination of the carbohydrate sequences and anomeric configurations, glycolipids were hydrolyzed with the following glycosidases: α-glucosidase (rice; Sigma) in 50 mM citrate buffer (pH 4.5), β-glucosidase (almond; Wako, Tokyo) in 50 mM citrate buffer (pH 6.5), and α-galactosidase (coffee bean; Sigma) in 50 mM citrate buffer (pH 4.5). Glycolipids (about 2 μg) together with 100 μg/μl sodium taurocholate were hydrolyzed by

incubation with the enzyme at 37°C for 1–16 h in a final volume of 100 μl, and the products were recovered by solvent partitioning with 100 μl of chloroform and 50 μl methanol, followed by 100 μl of methanol/water (1:1, by vol.), and were examined by TLC with chloroform/methanol/water (65:25:4, by vol.) and orcinol-sulfuric acid reagent for glycolipids, and with n-hexane/diethyl ether/acetic acid (80:30:4, by vol.) and cupric acetate-phosphoric acid reagent for DG [2].

Results

Lipids in bacteria The major phospholipids in LR, SE and SS were CL and PG, which closely migrated on a glass-coated TLC plate, but were clearly separated on a plastic-coated TLC plate (Fig. 1), and whose structures were characterized by negative ion FABMS spectrometry, which gave molecular ions $[M-H]^-$ for PG and $[M-2H]^-$ for CL (Figs. 2 and 3). As to glycolipids, MH-DG, DH-DG, TH-DG and TetH-DG were purified from LR, and their carbohydrate structures were determined to be Glcα1-3′DG, Galα1-2Glcα1-3′DG, Galα1-6Galα1-2Glcα1-3′DG and Galα1-6Galα1-6Galα1-2Glcα1-3′DG, respectively, which were identical with those in LJ and *L. intestinalis,* according to the procedures described above [2]. SE and SS contained MH-DG and DH-DG as neutral glycolipids, whose structures were also determined to be Glcβ1-3′DG and Glcβ1-6Glcβ1-3′DG, and Glcα1-3′DG, and Glcα1-2Glcα1-3′DG, respectively [2]. As shown in Fig. 1 and Table 1, DH-DG, as a major glycolipid in all bacteria, was contained in amounts equivalent to those of phospholipids.

Molecular species of phospholipids and glycolipids in bacteria Lipids purified from bacteria were methanolyzed and the resultant fatty acid methyl esters were analyzed by GC-MS. Their compositions were found to be essentially identical between phospholipids and glycolipids in a bacterial species-characteristic manner. Although the relative proportions of 16:0 and 18:1 of lipids of LR were higher and lower than those of LJ, respectively, cy19:0 was commonly contained in both LJ and LR as the major fatty acid, and the fatty acid compositions of all lipids in LR were similar to those of DH-DG in LJ and *L. intestinalis,* as reported previously [2]. As to hydroxy fatty acids (18h:0) in DH-DG from LJ and *L. casei* reported in our previous paper [2], since it was not detected, as molecular ions, on direct analysis of DH-DG by positive ion FABMS, it was concluded to be yielded from cy19:0 through methanolysis. While, all lipids from SE and SS were revealed to have the same fatty acid compositions as those of DH-DGs in our previous study [2].

On the basis of the fatty acid compositions of phospholipids and glycolipids on GC-MS, individual molecular species, as the combination of the fatty acids in DG moieties, were determined from their molecular ions, *i.e.* $[M+Na]^+$ for glycolipids, $[M-H]^-$ for PG and $[M-2H]^-$ for CL, by FABMS, and the relative ion intensities were found to coincide with the fatty acid compositions on GC-MS (Figs. 2 and 3). The major molecular ions, $[M+Na]^+$ at m/z 794 for MH-DG (Fig. 2A), $[M+Na]^+$ at m/z 956 for DH-DG (Fig. 2D), and $[M-H]^-$ at m/z 762, for PG (Fig. 3A) from LR corresponded to the molecular species with 16:0 and cy19:0-DG (Table 2), which differed from that 18:1 and cy19:0-containing DH-DG were the major molecular species in LJ and *L. intestinalis* [2]. Also, MH-DG, DH-DG and PG from SE gave major molecular ions at m/z 754, m/z 916 and m/z 722, respectively, corresponding to ai15:0- and ai17:0-containing structures (Figs. 2 and 3), which comprised about 40 % of the total molecular species of MH-DG, DH-DG and PG (Table 2). Similarly, the major molecular species of MH-DG, DH-DG and PG from SS were found to be constructed from 16:0- and 18:1-

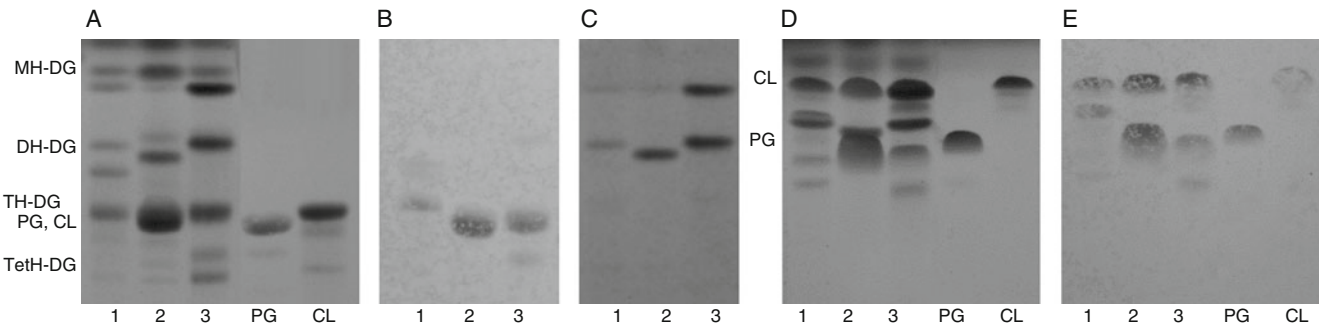

Fig. 1 TLC of lipids derived from LR (1), SE (2), and SS (3). Lipids, corresponding to 0.5 mg dry weight, were developed on glass-coated (**A**, **B** and **C**) and plastic-coated (D and E) TLC plates with chloroform/methanol /water (65:25:4, by vol.), and were detected with cupric acetate-phosphoric acid (**A** and **D**), Dittmer's reagent (**B** and **E**), and orcinol-sulfuric acid reagent (**C**)

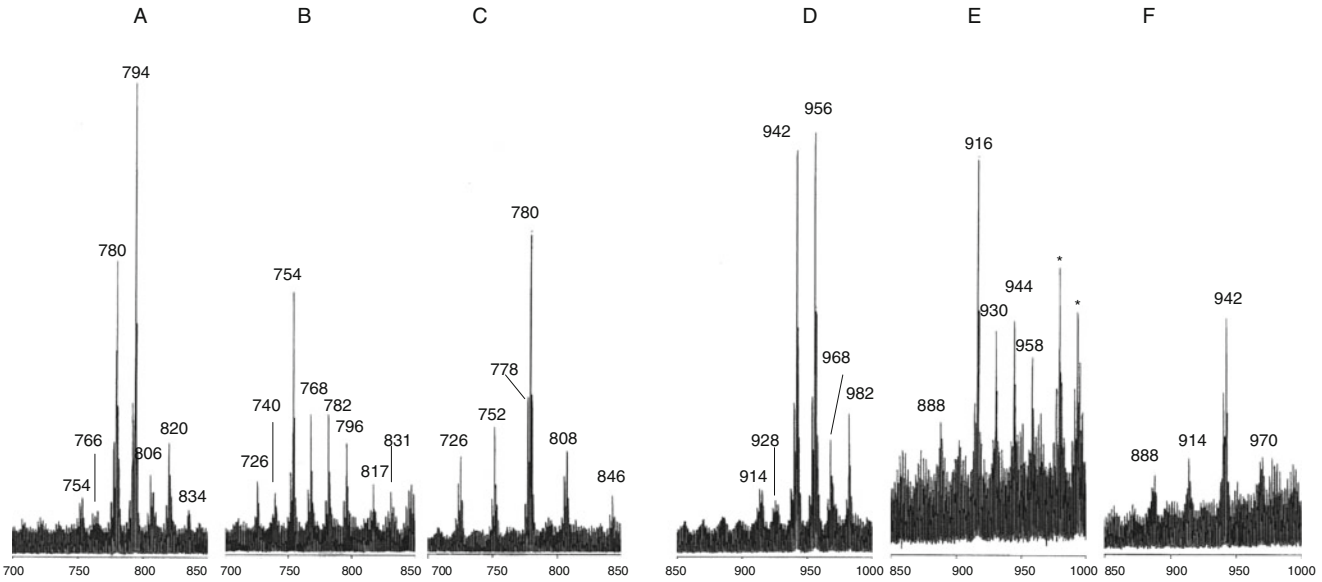

Fig. 2 Positive ion FABMS spectra of MH-DG (A to C) and DH-DGs (D to F).MH-DG and DH-DG from LR (**A** and **D**), from SE (**B** and **E**), and from SS (**C** and **F**) were analyzed by positive ion FABMS with triethanolamine as the matrix, and their molecular ions, $[M+Na]^+$, are represented in the figure. *, $[M+triethanolamine]^+$

containing structures, and to comprise 40–46 % of the total (Figs. 2 and 3, and Table 2). In addition, these molecular characteristics of the hydrophobic DG moieties of lipids in individual bacteria were retained in the molecular species of CL (Fig. 3 and Table 3). Thus, phospholipids and glycolipids in individual bacteria were revealed to be composed of the same DG, whose structure was distinct among bacterial species inhabiting different circumstances, *i.e.* the intestines, skin and oral cavity.

Antigenic lipids in bacteria Anti-LJ, anti-SE and anti-SS antisera prepared by immunization of rabbits with the respective bacteria exhibited titers of more than 1:10,000 on serum dilution on ELISA, and all of them were found to react with glycolipids, but not with phospholipids from the respective bacteria on TLC-immunostaining (Fig. 4). Anti-LJ antiserum reacted with DH-DG, TH-DG and TetH-DG, but not with MH-DG from LR. The relative reactivity of antiserum to DH-DG from LR was less than one-tenth of

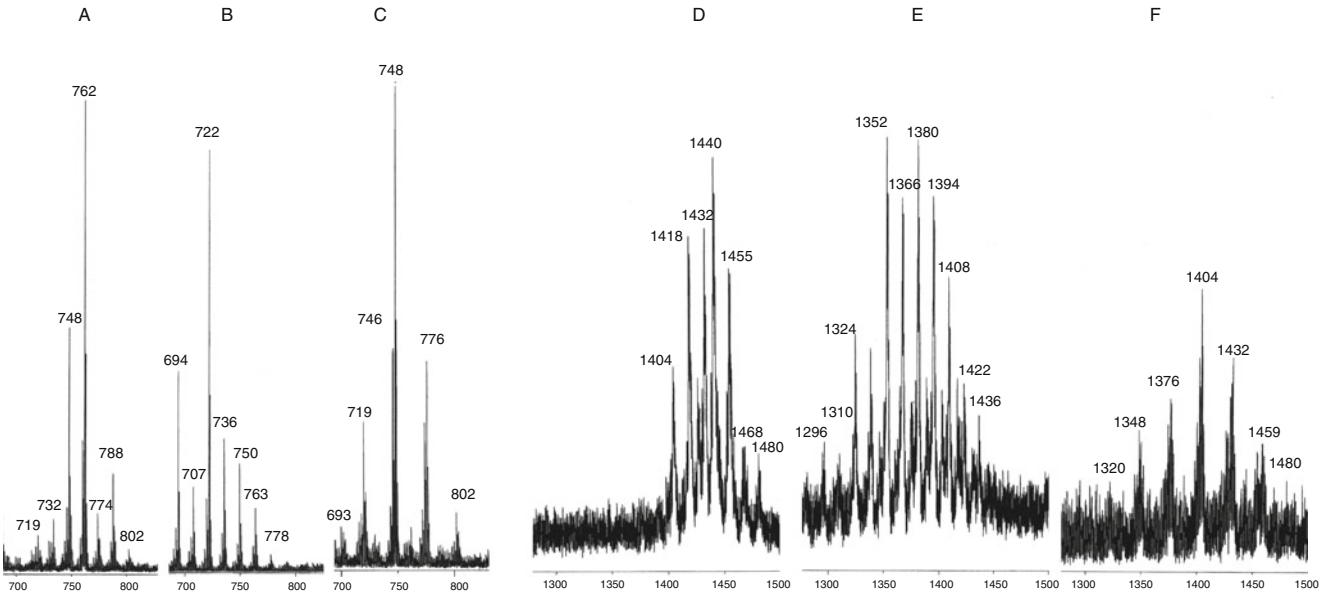

Fig. 3 Negative ion FABMS spectra of PG (**A** to **C**) and CL (**D** to **F**). PG and CL from LR (**A** and **D**), from SE (**B** and **E**), and from SS (**C** and **F**) were analyzed by negative ion FABMS with triethanolamine as the matrix, and their molecular ions, $[M-H]^-$ for PG and $[M-2H]^-$ for CL, are represented in the figure

Table 1 Amounts of lipids in *L. reuteri*, *S. epidermidis* and *S. salivalis* (μg/mg dried bacteria)

	L. reuteri	*S. epidermidis*	*S. salivalis*
CL	0.22±0.05	0.94±0.11	0.78±0.06
PG	0.43±0.05	1.87±0.15	0.78±0.08
Glcα1-3′DG	0.23±0.02	–	1.60±0.05
Glcβ1-3′DG	–	0.14±0.02	–
Galα1-2Glcα1-3′DG	0.31±0.01	–	–
Glcβ1-6Glcβ1-3′DG	–	0.79±0.02	–
Glcα1-2Glcα1-3′DG	–	–	1.22±0.03
Galα1-6Galα1-2Glcα1-3′DG	0.04±0.01	–	–
Galα1-6Galα1-6Galα1-2Glcα1-3′DG	0.06±0.01	–	–

that to TH-DG and TetH-DG from LR, indicating the strong antigenicity of TH-DG and TetH-DG in intestinal Lactobacilli, *i.e.* LR, LJ and *L. intestinalis* (Fig. 4). However, anti-SE antiserum preferentially reacted with DH-DG from SE, anti-SS antiserum cross-reacted with DH-DGs from SE and LR. On TLC-immunostaining, the relative reactivity of anti-SS antiserum to DH-DG from SS was estimated to be more than five times higher than those to DH-DGs from SE and LR, but anti-SS antiserum did not react at all with MH-DGs from all bacteria, or TH-DG and TetH-DG from LR (Fig. 4). In addition, both anti-SE and anti-SS antisera did not cross-react with glycosphingolipids containing Glcβ and Galα at their nonreducing terminals (Fig. 5). Accordingly, although anti-SS antiserum reacted with not only Glcα1-2Glcα-, but also Galα1-2Glcα- and Glcβ1-6Glcβ-structures, anti-LJ antiserum showed antigenic specificity toward the Galα1-2Glcα-, Galα1-6Galα1-2Glcα- and Galα1-6Galα1-6Galα1-2Glcα-structures in Lactobacillus species, and anti-SE antiserum toward the Glcβ1-6Glcβ-structure in Staphylococcus species. In fact, as shown in Fig. 6, anti-LJ antiserum reacted with glycolipids from LJ and *L. casei*, in which TH-DG and TetH-DG were selectively distributed among several Lactobacillus strains [2]. While, Glcβ1-6Glcβ1-3′DG, as a Staphylococcus species-specific antigen, was clearly detected using anti-SE antiserum in all Staphylococcus strains, *i.e.* SE from JCM and ATCC, and *S. aureus,* but not in Lactobacillus, Streptococcus or Escherichia (Fig. 6).

Table 2 Relative intensities of molecular ions from PG, MH-DG and DH-DG in *L. reuteri*, *S. epidermidis* and *S. salivalis*

	Moleculars pecies	PG		MH-DG		DH-DG	
		[M-H]-	%	[M+Na]+	%	[M+Na]+	%
L. reuteri	16:0 16:1	719	2.9	754	3.1	914	2.8
	14:0 cy19:0	732	4.2	766	1.6	928	1.9
	16:0 18:1	748	24.8	780	28.5	942	37.7
	16:0 cy19:0	762	50.3	794	47.4	956	38.9
	18:1 18:1	774	6.6	806	5.4	968	8.1
	18:1 cy19:0	788	9.5	820	12.1	982	10.6
	cy19:0 cy19:0	802	1.7	834	1.9	996	–
S.epidermidis	14:0 16:0	694	19.7	726	7.5	888	6.1
	ai15:0 16:0	707	7.8	740	5.6	902	3.8
	ai15:0 ai17:0	722	43.1	754	37.7	916	39.3
	16:0 ai17:0	736	12.9	768	18.0	930	17.9
	ai17:0 ai17:0	750	10.4	782	17.9	944	18.9
	ai17:0 18:0	763	6.1	796	13.3	958	14.0
S.salivalis	14:0 16:0	693	2.3	726	11.9	888	8.8
	16:0 16:1	719	15.8	752	15.3	914	11.0
	16:1 18:1	746	16.8	778	19.4	940	24.6
	16:0 18:1	748	46.5	780	42.6	942	40.8
	18:0 18:1	776	15.4	808	10.8	970	8.5
	18:1 20:1	802	3.2	–	–	996	6.3

cy, cyclopropane; ai, anteiso

Table 3 Relative intensities of molecular ions from caldiolipin in *L. reuteri*, *S. epidermidis* and *S. salivalis* on FABMS

	Molecular species	[M-2H]-	%
L. reuteri	16:0 16:0 18:1 18:1	1404	9.6
	16:0 16:0 18:1 cy19:0	1418	17.2
	14:0 18:1 cy19:0 cy19:0	1428	9.0
	16:0 16:0 cy19:0 cy19:0, 16:0 18:0 18:1 18:1	1432	17.9
	16:0 18:1 18:1 cy19:0	1440	22.3
	16:0 18:1 18:1 18:1	1455	15.4
	18:1 18:1 18:1 cy19:0	1468	4.5
	18:1 18:1 cy19:1 cy19:0	1480	4.1
S. epidermidis	14:0 14:0 16:0 16:0	1296	3.4
	14:0 ai15:0 16:0 16:0	1310	2.9
	ai15:0 ai15:0 16:0 ai17:0	1324	7.7
	ai15:0 ai15:0 16:0 ai17:0	1338	7.3
	ai15:0 ai15:0 ai17:0 ai17:0	1352	15.8
	ai15:0 16:0 ai17:0 ai17:0	1366	13.4
	ai15:0 ai17:0 ai17:0 ai17:0, ai15:0 16:0 16:0 18:0, 16:0 16:0 ai17:0 ai17:0	1380	15.8
	ai15:0 ai17:0 ai17:0 18:0, 16:0 ai17:0 ai7:0 ai17:0,	1394	13.4
	16:0 ai17:0 ai17:0 18:0, ai17:0 ai17:0 ai17:0 ai17:0	1408	10.2
	ai17:0 ai17:0 ai17:0 18:0	1422	5.7
	ai17:0 ai17:0 18:0 18:0	1436	4.4
S. salivalis	14:0 14:0 16:0 16:1	1292	6.6
	14:0 16:0 16:0 16:1	1320	6.6
	16:0 16:0 16:1 16:1, 14:0 16:0 16:1 18:1	1348	12.2
	16:0 16:0 16:1 18:1	1376	15.8
	16:0 16:0 18:1 18:1, 16:0 16:1 18:0 18:1	1404	27.7
	16:0 16:0 18:1 18:1	1432	20.2
	16:0 18:1 18:1 20:1	1459	10.9

cy, cyclopropane; ai, anteiso

Antibodies toward bacterial glycolipids in human sera Human sera of blood groups A, B, AB and O were reacted with purified bacterial glycolipids (0.5 μg/well), *i.e.* Galα1-2Glc1-3′DG, Glcβ1-6β1-3′DG, Glcα1-2Glcα1-3′DG, Galα1-6Galα1-2Glcα1-3′DG and Galα1-6Galα1-6Galα1-2Glcα1-3′DG, on an ELISA plate, and about 75 % of the sera, irrespective of the blood group, were found to contain IgM antibodies toward TH-DG and TetH-DG, exhibiting optical densities of more than 0.3 (Fig. 7). In addition, TLC-immunostaining of these glycolipids with human sera of blood groups O and AB clearly demonstrated the presence of IgM antibodies toward TH-DG and TetH-DG (Fig. 8). However, antibodies toward DH-DGs from LR, SE and SS were not detected on ELISA or TLC-immunostaining (Figs. 7 and 8), indicating that the antigenic determinant of human IgM is the Galα1-6Galα- structure.

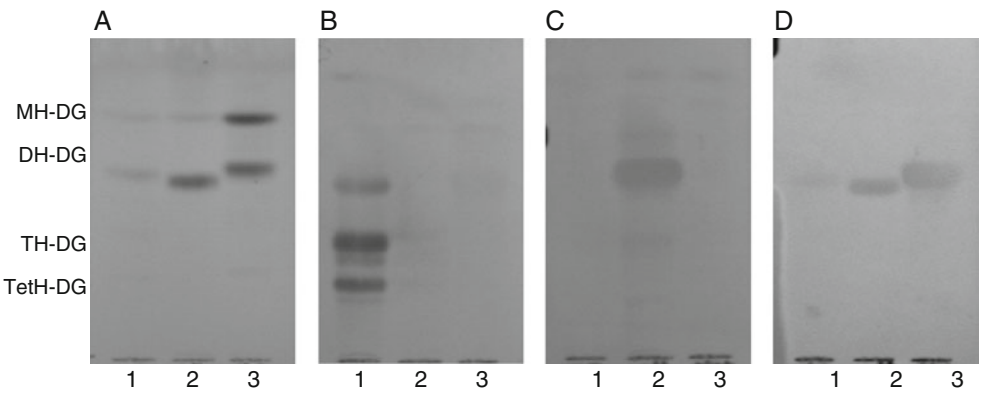

Fig. 4 TLC and TLC-immunostaining of lipids from LR (1), SE (2), and SS (3).Lipids, corresponding to 1 mg dried bacteria, were developed on TLC plates with chloroform/methanol/water (65:25:4, by vol.), and the spots were visualized with orcinol-sulfuric acid reagent (**A**), and anti-LJ (**B**), anti-SE (**C**), and anti-SS (**D**) antisera

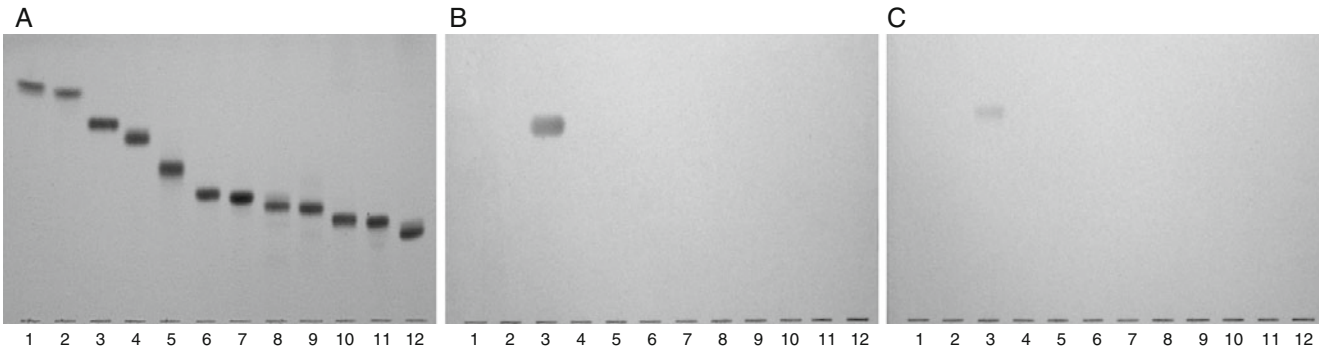

Fig. 5 TLC and TLC-immunostaining of DH-DGs from SE and SS, and glycosphingolipids.About 2–4 μg of DH-DGs from SE (A3 and B3) and SS (C3), and glycosphingolipids were developed on TLC plates with chloroform/methanol/0.5 % $CaCl_2$ in water (55:45:10, by vol.), and the spots were visualized with orcinol-sulfuric acid reagent (**A**), and anti-SE (**B**) and anti-SS (**C**) antisera. 1, GlcCer; 2, GalCer; 4, LacCer; 5, Gb_3Cer; 6, Gg_3Cer; 7, Gb_4Cer; 8, nLc_4Cer; 9, Lc_4Cer; 10, $VI^3GalNAc\alpha$-Gb_4Cer; 11, $IV^3Gal\alpha nLc_4Cer$; 12, Gg_4Cer

Discussion

Judging from the space-filling model of phospholipids with unsaturated fatty acids, the three dimensional structures of CL and MH-DG with smaller polar head groups are conical in shape and, on the contrary, those of PG and DH-DG with larger head groups are reverse conical in shape, assuming that PG and DH-DG, and CL and MH-DG are distributed in the outer and inner leaflets of the lipid bilayer, respectively [15]. However, one can suggest that all glycolipids including MH-DG are in the outer leaflet like those in animal cells, in which glycolipids are distributed in the raft structures to hold membrane proteins on the liquid crystals of phospholipids and cholesterol [16]. The molecular configuration of glycolipids in membranes is known to be dependent on their hydrophobic moieties, *i.e.* DG and ceramides. Although PA is a common precursor for the synthesis of phospholipids and glycolipids, they are synthesized through different pathways, *i.e.* glycolipids through transfer of carbohydrates to DG from PA, PG through conversion of PA to CDP-DG, and dephosphorylation of PG-phosphate from glycerol-3-phosphate and CDP-DG, and CL from two molecules of PG [17]. In general, enzymes in these pathways exhibit substrate specificity toward a narrow range of molecular species, resulting in different molecular species in substrates and products, and also remodeling through deacylation and reacylation occurs at individual steps to modify the molecular species. In particular, remodeling of CL has been proven to be involved in the onset of Barth syndrome [18, 19]. However, as shown in this paper, glycolipids and phospholipids in three bacteria were constructed of similar molecular species of DG moieties without large structural modifications, indicating that remodeling does not occur significantly between glycolipids and phospholipids in bacteria, and that both lipids are distributed symmetrically. Accordingly, the molecular species of DG moieties in glycolipids and phospholipids, rather than their compositions, should be responsible for the physicochemical properties of biomembranes for bacteria to adapt to several circumstances. For instance, from the space filling model of fatty acids [15], one can suggest that DG with ai15:0 and ai17:0 firmly connects the outer and inner leaflets through their anteiso groups to form a solid membrane with strong water-holding activity as to SE in skin, and that with cyclopropane ring-

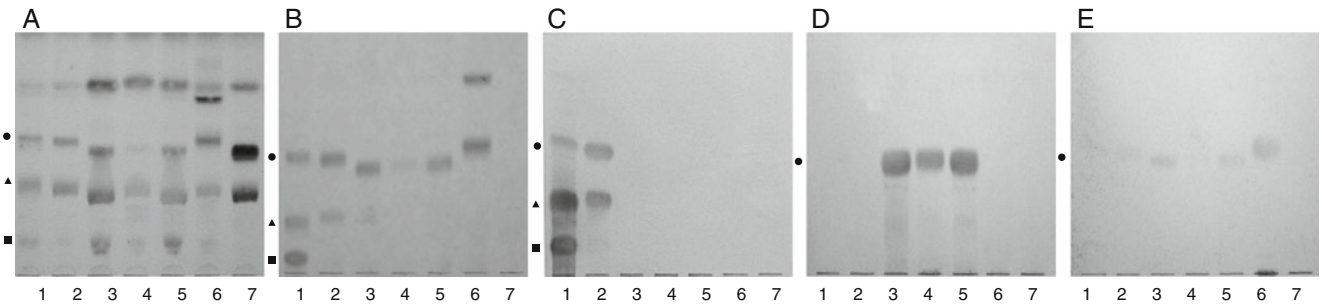

Fig. 6 TLC and TLC-immunostaining of lipids from several bacteria. Lipids, corresponding to 0.5 mg dried bacteria, were developed on TLC plates with chloroform/methanol/water (65:25:4, by vol.), and the spots were detected with cupric acetate-phosphoric acid (**A**), orcinol-sulfuric acid reagent (**B**), and anti-LJ (**C**), anti-SE (**D**) and anti-SS antisera (**E**). 1, LJ; 2. *L. casei*; 3, *S. aureus* (ATCC860); 4, SE (JCM2414); 5, SE (ATCC 866); 6, SS; 7, *E.coli*. Symbols beside each TLC plate indicate the positions of DH-DG (●), TH-DG (▲), and TetH-DHG (■), respectively

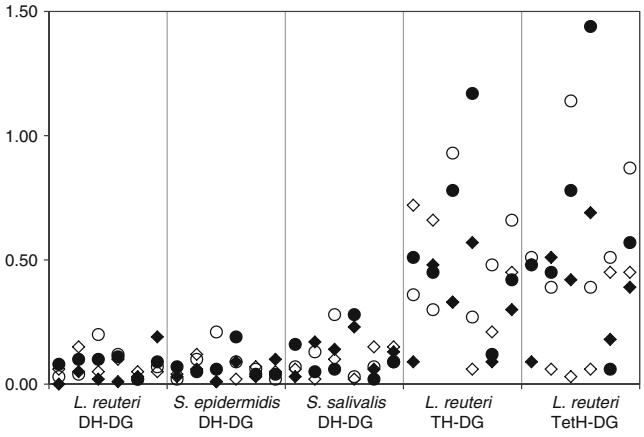

Fig. 7 Reactivities of human sera with bacterial glycolipids on ELISA. Human sera diluted 1:100 with 1 % BSA in PBS were reacted with Galα1-2Glc1-3′DG (DH-DG from LR), Glcβ1-6β1-3′DG (DH-DG from SE), Glcα1-2Glcα1-3′DG (DH-DG from SS), Galα1-6Galα1-2Glcα1-3′DG (TH-DG from LR), and Galα1-6Galα1-6Galα1-2Glcα1-3′DG (TetH-DG from LR) on a ELISA plate as described in the text. Blood groups: ◊, A; ♦, B; ○, AB; ●, O

containing fatty acids provides stability under the acidic conditions on exposure to lactic acid secreted from Lactobacillus species than that with unsaturated fatty acids, for LR in the intestines. While, SS inhabiting in the oral cavity under rather milder conditions than those in the skin and intestines carried molecular species rich in 16:0- and 18:1-, and 16:1- and 18:1-containing DG.

On the other hand, in comparison to biomembranes with choline-, ethanolamine- and serine-containing phospholipids in animal cells, the polar head groups of phospholipids, *i.e.* glycerol phosphate of PG, glycerol bisphosphate of CL, and mono- and di-hexoses of MH-DG, and DH-DG, should develop a hydrogen-bond network on the outer and inner surfaces of biomembranes through their hydrogen-donor groups, and MH-DG and DH-DG rich in hydrogen-donor groups might play a role by holding membrane proteins and regulating their activities, like glycolipids in animal cells [16].

Another function of bacterial glycolipids is known to be the holding of teichoic acids as the major constituents of cell walls in Gram-positive bacteria. By extraction from Streptococcus species with phenol-containing solvents, lipoteichoic acids with Glcα1-2Glcα1-3′DG have been isolated, and their structures have been determined to be poly(glycerol-1,3-phosphate)-6Glcα1-2Glcα1-3′DG and poly(6Galα1-6Galα1-3″, Galα1-2″, glycerol-1″-phosphate)-6Glcα1-2Glc(6-fatty acid)α1-3′DG [20, 21], but they were not obtained from SS on extraction with chloroform and methanol-containing solvents due to their polar properties. Consequently, the majority of DH-DG was shown to be present without teichoic acids as the membrane constituents in LR, SE and SS.

As to the antigenic properties of bacterial glycolipids, antibodies toward DH-DG, TH-DG and TetH-DG were effectively generated on direct immunization of rabbits with the bacteria. Also, bacterial aggregation with antibacterial antiserum was attenuated by absorption of antiserum with the respective DH-DG, indicating that glycolipids are partly exposed on the cell wall, although their exact configuration on the cell wall is obscure (Iwamori *et al.*, unpublished observation).

Since the structures of DH-DGs are bacterial species-specific, *i.e.* Galα1-2Glcα1-3′DG for Lactobacillus, Glcβ1-6Glcβ1-3′DG for Staphylococcus and Glcα1-2Glcα1-3′DG for Streptococcus, and their amounts are significantly high, they function not only as antigenic determinants for immune recognition by the host animals, but also as a landmark for mutual recognition among individual bacterial species [2, 3, 22, 23]. As to other bacterial species, the following DH-DGs have been reported in the literature, Galβ1-2Galβ1-3′DG for Bifidobacterium, Glcβ1-4glucuronic acidα1-3′DG for Pseudomonas, Galα1-4galacturonic acidα1-3′DG for

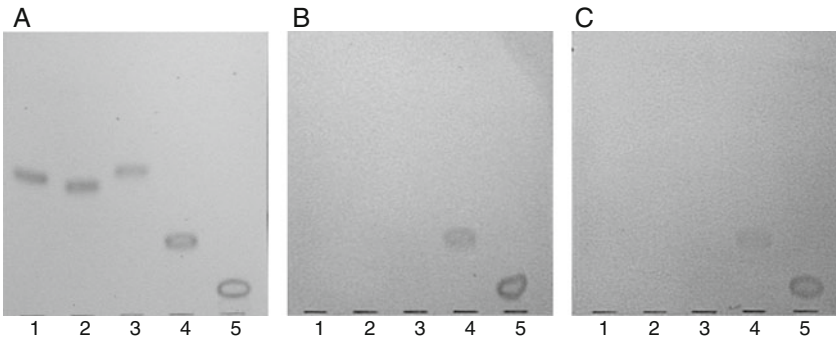

Fig. 8 TLC and TLC-immunostaining of bacterial glycolipids. About 1 μg of Galα1-2Glc1-3′DG (1), Glcβ1-6Glcβ1-3′DG (2), Glcα1-2Glcα1-3′DG (3), Galα1-6Galα1-2Glcα1-3′DG (4), and Galα1-6Gal1-6Galα1-2Glcα1-3′DG (5) were developed on TLC plates with chloroform/methanol/water (65:25:4, by vol.), and then detected with orcinol-sulfuric acid reagent (**A**), human serum (blood group O, 1:100) (**B**), and human serum (blood group AB, 1:100) (**C**)

Streptomyces, Manα1-3Manα1-3′DG for Micrococcus and Galβ1-6Galβ1-3′DG for Arthrobacter [20, 24], and these structures must generate antibodies on immunization of rabbits.

On comparison of the cross-reactivities of antisera toward LJ, SE and SS, it was found they did not react with MH-DG or glycosphingolipids containing the same carbohydrate structures at the nonreducing terminals at all, but reacted with DH-DG, TH-DG and TetH-DG from LR, and DH-DG from SE and DH-DG from SS as their preferable antigens, respectively, indicating that dihexaosyl groups are the minimum antigenic determinants. Anti-SE antiserum only reacted with Glcβ1-6Glcβ in SE, but although anti-LJ and anti-SS antiserum strongly reacted with the respective bacterial antigens, *i.e.* Galα1-2Glcα in LR and Glcα1-2Glcα in SS, respectively, they also cross-reacted with each other at intensities of less than one-fifth as to their proper antigens, mainly due to the similarity in the three-dimensional structures of their dihexaosyl groups. The cross-reactivity of bacterial glycolipids with antibacterial antiserum might be relevant to the similarity of bacterial species as to the rabbit immune system. In human sera, irrespective of the ABO blood group, antibodies against TH-DG and TetH-DG from LR were found to be present at a significantly high frequency. Since Galα at the nonreducing terminals of TH-DG and TetH-DG is a part of the blood group B determinant, anti-LJ antiserum was initially thought to react with blood group B glycosphingolipids, it did not react with them at all [2, 3], indicating that intestinal Lactobacilli are not involved in the production of anti-blood group B antibodies. Also, antibodies toward the Galα1-3Galβ1-4GlcNAcβ-structure, which is widely distributed in several animal species including cow, pig and rabbit, are frequently detected in normal human sera and thus function as an obstacle for xenogeneic transplantation [25], and their production was found not to be due to an immune reaction toward intestinal Lactobacilli, because of no reaction of anti-LJ antiserum to Galα-containing glycosphingolipids. Consequently, antibodies in human sera seemed to react with the Galα1-6Galα- structure of TH-DG and TetH-DG in several strains of intestinal Lactobacilli, with sufficiently high specificity, probably for prevention of their invasion to internal bodies from the mucosal layer. One can suggest that Lactobacilli with these antigenic glycolipids act as symbiotic bacteria in the intestines, and no immune reaction is required to bacteria in the skin or oral cavity of humans. Thus, glycolipids in symbiotic bacteria in human different tissues were revealed to be composed of unique structures as to both oligosaccharides and DG moieties, which are thought to be prerequisite for the establishment of symbiosis in different tissues.

References

1. IUPAC-IUB: Commission on biochemical nomenclature: the nomenclature of lipids. Eur. J. Biochem. **179**, 11–21 (1977)
2. Iwamori, M., Sakai, A., Minamimoto, N., Iwamori, Y., Tanaka, K., Aoki, D., Adachi, S., Nomura, T.: Characterization of novel glycolipid antigens with an α-galactose epitope in lactobacilli detected with rabbit anti-Lactobacillus antiserum and occurrence of antibodies against them in human sera. J. Biochem. **150**, 515–523 (2011)
3. Iwamori, M., Iwamori, Y., Adachi, S., Nomura, T.: 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. Glycoconj. J. **28**, 21–30 (2011)
4. Iwamori, M., Shibagaki, T., Nakata, Y., Adachi, S., Nomura, T.: Distribution of receptor glycolipids for Lactobacilli in murine digestive tract and production of antibodies cross-reactive with them by immunization of rabbits with Lactobacilli. J. Biochem. **146**, 185–191 (2009)
5. Tamai, Y., Matsukawa, S., Satake, M.: Lipid composition of nerve cell perikarya. Brain Res. **26**, 149–157 (1971)
6. Hamberger, A., Svennerholm, L.: Composition of gangliosides and phospholipids of neuronal and glial cell enriched fractions. J. Neurochem. **18**, 1821–9 (1971)
7. Odutuga, A.A., Carey, E.M., Prout, R.E.: Changes in the lipid and fatty acid composition of developing rabbit brain. Biochim. Biophys. Acta **316**, 115–123 (1973)
8. Pewzner-Jung, Y., Ben-Dor, S., Futerman, A.H.: When do Lasses (longevity assurance genes) become CerS (ceramide synthases)?: Insights into the regulation of ceramide synthesis. J. Biol. Chem. **281**, 25001–25005 (2006)
9. Yohe, H.C., Roark, D.E., Rosenberg, A.: C20-sphingosine as a determining factor in aggregation of gangliosides. J. Biol. Chem. **251**, 7083–7087 (1976)
10. Lands, W.E.: Metabolism of glycerolipides; a comparison of lecithin and triglyceride synthesis. J. Biol. Chem. **231**, 883–888 (1958)
11. Lewin, T.M., Wang, P., Coleman, R.A.: Analysis of amino acid motifs diagnostic for the sn-glycerol-3-phosphate acyltransferase reaction. Biochemistry **38**, 764–771 (1999)
12. Bartlett, G.R.: Phosphorus assay in column chromatography. J. Biol. Chem. **234**, 466–468 (1959)
13. Folch, J., Lees, M.: A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226**, 497–509 (1957)
14. Iwamori, M., Takamizawa, K., Momoeda, M., Iwamori, Y., Taketani, Y.: Gangliosides in human, cow and goat milk, and their abilities as to neutralization of cholera toxin and botulinum type A neurotoxin. Glycoconj. J. **25**, 675–683 (2008)
15. Harrison, R., Lunt, G.G.: Biological membranes, Blackie, Glasgow/London (1980)
16. Füllekrug, J., Simons, K.: Lipid rafts and apical membrane traffic. Ann. N. Y. Acad. Sci. **1014**, 164–169 (2004)
17. Hirschberg, C.B., Kennedy, E.P.: Mechanism of the enzymatic synthesis of cardiolipin in Escherichia coli. Proc. Natl. Acad. Sci. U. S. A. **69**, 648–651 (1972)
18. Testet, E., Laroche-Traineau, J., Noubhani, A., Coulon, D., Bunoust, O., Camougrand, N., Manon, S., Lessire, R., Bessoule, J.J.: Ypr140wp, 'the yeast tafazzin', displays a mitochondrial lysophosphatidylcholine (lyso-PC) acyltransferase activity related to triacylglycerol and mitochondrial lipid synthesis. Biochem. J. **387**, 617–626 (2005)
19. Xu, Y., Condell, M., Plesken, H., Edelman-Novemsky, I., Ma, J., Ren, M., Schlame, M.: A Drosophila model of Barth syndrome. Proc. Natl. Acad. Sci. U. S. A. **103**, 11584–11588 (2006)
20. Shaw, N., Baddiley, J.: Structure and distribution of glycosyl diglycerides in bacteria. Nature **217**, 142–144 (1968)

21. Koch, H.U., Fischer, W.: Acyldiglucosyldiacylglycerol-containing lipoteichoic acid with a poly(3-O-galabiosyl-2-O-galactosyl-sn-glycero-1-phosphate) chain from Streptococcus lactis Kiel 42172. Biochemistry **17**, 5275–5281 (1978)
22. Smith, P.F.: Lipid composition of Mycoplasma neurolyticum. J. Bacteriol. **112**, 554–558 (1972)
23. Sugiyama, T., Smith, P.F., Langworthy, T.A., Mayberry, W.R.: Immunological analysis of glycolipids and lipopolysaccharides derived from various mycoplasmas. Infect. Immun. **10**, 1273–1279 (1974)
24. Shaw, N.: Bacterial glycolipids. Bacteriol. Rev. **34**, 365–377 (1970)
25. Tanemura, M., Miyagawa, S., Koyota, S., Koma, M., Matsuda, H., Tsuji, S., Shirakura, R., Taniguchi, N.: Reduction of the major swine xenoantigen, the alpha-galactosyl epitope by transfection of the alpha2,3-sialyltransferase gene. J. Biol. Chem. **273**, 16421–16425 (1998)