Characterization of a *Lactobacillus gasseri* JCM 1131[T] Lipoteichoic Acid with a Novel Glycolipid Anchor Structure

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We determined the chemical structure of lipoteichoic acid (LTA) from *Lactobacillus gasseri* JCM 1131[T]. The repeating unit was comprised of glycerolphosphate and 2-alanylglycerolphosphate. The glycolipid anchor was tetrahexosylglycerol with two or three acyl groups. To our knowledge, this is the first demonstration of a tetrahexose structure in an LTA glycolipid anchor.

Clarification of the cell envelope structure of probiotic lactic acid bacteria is important for understanding their host-microbe interactions. In a comparison of the genomes from 12 species of lactobacilli, large proportions (19.9 to 29.3%) of the putative total proteins were estimated to be cell surface proteins, including membrane proteins (1). In particular, the genomes of *Lactobacillus acidophilus* group members (e.g., *L. acidophilus*, *Lactobacillus johnsonii*, and *Lactobacillus gasseri*), which are often used as probiotics, encode a large number of LPXTG-motif proteins, approximately half of which are estimated to have mucus-binding properties (1). Peptidoglycan and teichoic acids are also important molecules, as they are recognized by host immune cells.

Teichoic acids are classified as wall teichoic acid (WTA) and lipoteichoic acid (LTA). WTA links covalently to the *N*-acetylmuramic acid residues in cell wall peptidoglycan, while LTA is anchored to the cell membrane through its glycolipid moiety. In particular, LTA has been shown to act as a ligand for Toll-like receptor 2 on host immune cells (2) and to induce production of proinflammatory cytokines (3). LTA is also suggested to work as an adhesion molecule with human intestinal epithelial cells (4). Fibronectin may be a host receptor for LTA (5). However, the structural information regarding LTA is insufficient, given the complex nature of this molecule.

LTA is an anionic polymer comprised of a repeating glycerophosphate (GroP) backbone that is connected to a glycolipid (in many cases, dihexosyldiacylglycerol) through a phosphodiester linkage. The C-2 hydroxyl group of the GroP residue is often substituted by D-alanine and/or hexoses. Strain-level variation has been detected in both the degree of polymerization and the substitution ratio of the repeating GroP unit (6, 7).

Within the abovementioned *L. acidophilus* group members, structural information on LTA is limited and information is completely lacking for *L. gasseri*, although this species is often used as a probiotic. *L. gasseri* has been isolated not only from the intestine but also from the oral cavity, vagina, urine, and blood, suggesting a strong association between this bacterium and the human body. Thus, the structure of LTA from *L. gasseri* needs to be clarified to promote our understanding of the interaction of this bacterium with the host. Therefore, in this study, we determined the chemical structure of LTA from *L. gasseri* JCM 1131[T].

L. gasseri JCM 1131[T] was grown to log phase in 0.5× Difco Lactobacilli MRS broth (Becton, Dickinson and Co., Franklin Lakes, NJ). The cells were collected and disrupted, and the LTA was purified by *n*-butanol extraction followed by hydrophobic interaction chromatography (HIC) as described previously with some modifications (8). As a result, 38 mg of LTA was obtained from 25 g of wet cells. The repeating unit of LTA was analyzed with one- and two-dimensional nuclear magnetic resonance (NMR) spectroscopy. The glycolipid anchor fraction of LTA was prepared by treatment with 98% (vol/vol) acetic acid at 100°C for 3 h. The carbohydrate portion of the glycolipid anchor was obtained via deacylation by treatment with 20% (wt/vol) ammonia at room temperature for 12 h. The glycolipid anchor and its carbohydrate portion were analyzed with matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), which was performed as described previously, with some modifications (9, 10). The chemical composition of the glycolipid anchor, including anchor sugars and fatty acids, was analyzed with gas chromatography (GC) as described previously (11) (see the supplemental material for detailed descriptions of all these experimental conditions).

Structure of the repeating unit from *L. gasseri* JCM 1131[T] LTA. The structure of the repeating unit was determined by one- and two-dimensional NMR analyses. The peaks detected by ^{1}H NMR and ^{13}C distortionless enhancement by polarization transfer with an angle parameter of 135° (DEPT-135) NMR (see Fig. S1A, B, and C in the supplemental material) were attributable to the GroP residue and the 2-alanyl-GroP (2-AlaGroP) residue with a substitution at the C-2 hydroxyl group of glycerol to D-alanine (Table 1), as deduced from previous reports (9, 10, 12). No hexoses were detected as substituents. The assignments were supported by correlations with the results of correlation spectroscopy

TABLE 1 Assignments for the ^{1}H and ^{13}C DEPT-135 NMR spectra of the repeating-unit region of *L. gasseri* JCM 1131^T LTA

Residue	Proton (^{1}H)	δ (ppm)	Intensity^a	Carbon (^{13}C)	δ (ppm)
Unsubstituted glycerol	H-1, 3	3.90	1.38H (2H × 0.69)	C-1, 3	68.98
		3.96	1.38H (2H × 0.69)		
	H-2	4.05	0.69H (1H × 0.69)	C-2	72.32
Alanine-substituted glycerol	H-1, 3	4.11	1.24H (4H × 0.31)	C-1, 3	66.43
	H-2	5.40	0.31H (1H × 0.31)	C-2	77.01
Substituted alanine				C-1	172.84^b
	H-2	4.29	0.31H (1H × 0.31)	C-2	51.70
	H-3	1.63	0.93H (3H × 0.31)	C-3	18.08

^{a} The intensity was adjusted by taking a proton of one repeating unit as 1.
^{b} This peak was detected by heteronuclear multiple-bond correlation.

(COSY) (see Fig. S1A). The ratio of D-alanine substitution on the GroP residue was estimated to be 31% based on the intensity of the peaks in the ^{1}H NMR spectrum (Table 1). The average number of repeating units was estimated to be 20 to 30 according to the ratio of the peak area of protons in the GroP residue and protons bound to the carbons next to the double-bonded carbons in the unsaturated fatty acid residues (δ, 1.7 to 2.2 ppm) in the ^{1}H NMR spectrum.

The typical LTA of Gram-positive bacteria has been reported to contain a GroP backbone with frequent substitution of the C-2 hydroxyl groups by D-alanine and/or hexoses such as glucose, galactose, and *N*-acetylglucosamine (6). *L. gasseri* JCM 1131^T LTA also possesses a poly-GroP backbone with D-alanine substitution. Thus, *L. gasseri* JCM 1131^T showed a typical repeating-unit structure. The GroP repeating-unit structures in other lactobacilli have also been reported. In *Lactobacillus delbrueckii* subsp. *lactis* strains ATCC 15808, Ads-5, and LL78 (7); *Lactobacillus rhamnosus* GG (13); *Lactobacillus reuteri* 100-23 (14); and *Lactobacillus plantarum* NCIMB 8826 (15), 27 to 79% of the GroP residues are substituted with D-alanine. Among these, a hexose substitution (in all cases, glucose) was detected in *L. delbrueckii* subsp. *lactis* strains ATCC 15808 and LL78 and in *L. reuteri* 100-23 at 3 to 27%.

Structure of the glycolipid anchor from *L. gasseri* JCM 1131^T LTA. MALDI-TOF MS of the carbohydrate portion of the glycolipid anchor gave a peak at *m/z* 763.41, which was attributable to the $(M + Na)^+$ molecular ion of tetrahexosylglycerol (Fig. 1A). The tetrahexose was found to be composed of galactose and glucose at a molar ratio of 3:1 by GC. The fatty acid composition of LTA was determined by GC. Oleic acid [$C_{18:1}$(n-9)] was a major constituent, representing 70.0% of the total fatty acids. In addition, palmitic acid ($C_{16:0}$, 18.5%) and stearic acid ($C_{18:0}$, 7.3%) were detected. MALDI-TOF MS of the glycolipid anchor gave peaks which were divided into two groups with low (group 1) and high (group 2) molecular masses (Fig. 1B). These lines of evidence strongly suggest that the peaks in groups 1 and 2 were attributable to tetrahexosyldiacylglycerol (Hex$_4$DAG) and acyltetrahexosyldiacylglycerol (acylHex$_4$DAG), respectively, although the presence of acyltetrahexosylmonoacylglycerol cannot be excluded (16). For example, the peaks at *m/z* 1,292.01 and *m/z* 1,265.97 were attributable to Hex$_4$DAG containing $C_{18:1}$(n-9)/$C_{18:1}$(n-9) and $C_{18:1}$(n-9)/$C_{16:0}$, respectively. The peaks at *m/z* 1,557.40 and *m/z* 1,530.40 were attributable to acylHex$_4$DAG containing $C_{18:1}$(n-9)/$C_{18:1}$(n-9)/$C_{18:1}$(n-9) and $C_{18:1}$(n-9)/$C_{18:1}$(n-9)/$C_{16:0}$, respectively. Similar peak groups corresponding to trihexosyl-

diacylglycerol (Hex$_3$DAG) and acyltrihexosyldiacylglycerol (acylHex$_3$DAG) have been reported in *L. plantarum* LTA (9). The peak assignments for *L. gasseri* JCM 1131^T LTA were supported by a difference of 162 in molecular mass, corresponding to one hexose residue, compared to the mass spectrum of *L. plantarum* LTA (9). No equivalent peaks corresponding to Hex$_3$ structures were observed in *L. gasseri* JCM 1131^T (Fig. 1B).

The glycolipid anchor structures found in *L. gasseri* JCM 1131^T LTA, Hex$_4$DAG and acylHex$_4$DAG, are unique. To our knowledge, a Hex$_4$ structure has not been reported in a glycolipid anchor of LTA. Hex$_2$ structures are the most common in Gram-positive bacteria, including many staphylococci, bacilli, and streptococci. The LTA glycolipid anchor structures reported to date in four species of lactobacilli, *L. plantarum* KCTC 10887BP (9), *Lactobacillus helveticus* DSM 20075^T (17), *L. rhamnosus* DSM 20021^T (formerly *Lactobacillus casei*) (18), and *Lactobacillus pentosus* DSM 20314^T (formerly *L. plantarum*) (19), have been identified as
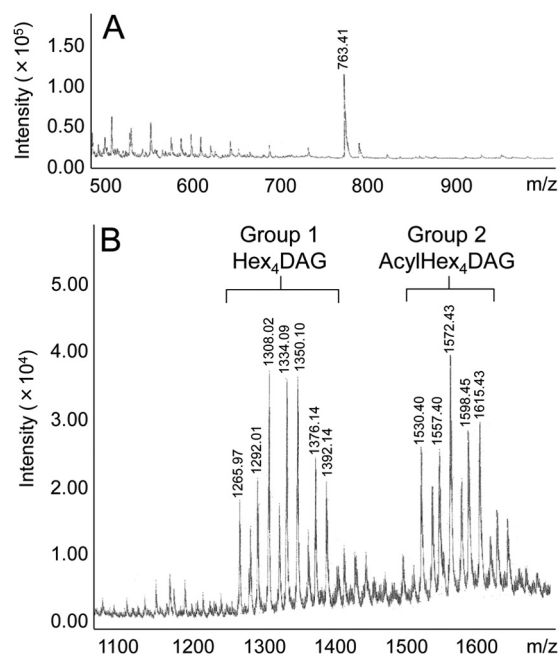

FIG 1 MALDI-TOF mass spectra of the glycolipid anchor fraction in *L. gasseri* JCM 1131^T LTA. (A) Carbohydrate portion. (B) Glycolipid anchor.
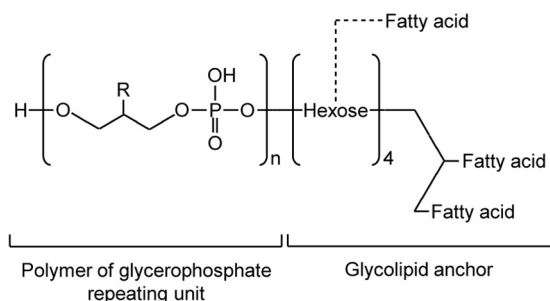
FIG 2 Putative chemical structure of *L. gasseri* JCM 1131^T LTA. For R, hydroxyl and D-alanyl groups were found at a ratio of 69:31. The hexoses identified were galactose and glucose at a ratio of 3:1. The fatty acids were oleic acid (70%), palmitic acid (19%), stearic acid (7%), and others (4%). $n = 20$ to 30.

Hex$_3$. The uniqueness of the Hex$_4$ structure found in *L. gasseri* JCM 1131^T might have some influence on the nature of the cell surface, leading to specific biological activities compared to those of other lactobacilli. The sugar composition was found to be glucose and galactose, which is common to the Hex$_3$ structure of LTA in the abovementioned four species of lactobacilli. On the other hand, the Hex$_2$ structure reported in many Gram-positive bacteria is composed exclusively of glucose.

Furthermore, the structure of acylHex$_4$DAG is unique in that it has three acyl groups. In most cases, the number of acyl groups has been reported to be two in Gram-positive bacteria. The distribution of glycolipid anchors having three acyl groups is relatively limited; it includes the abovementioned four species of lactobacilli and some lactococci, including *Lactococcus lactis* subsp. *lactis* NCDO 712 (formerly *Streptococcus lactis*) (20) and *Lactococcus garvieae* Kiel 42172 (formerly *S. lactis*) (21). Acyl groups are an important determinant of host receptor recognition, namely, Toll-like receptors (22). Thus, the number of acyl groups has a marked influence on the host-microbe interaction. For example, a loss of one acyl group from a two-acyl-group-type glycolipid anchor of LTA dramatically reduced cytokine production in *in vitro* cell culture experiments (23). Thus, the biological activity of acylHex$_4$DAG in the host-microbe interaction seems interesting.

The fatty acid composition of the glycolipid anchor in *L. gasseri* JCM 1131^T LTA was found to comprise 70% C$_{18:1}$(n-9) and 19% C$_{16:0}$, similar to the values reported for *L. rhamnosus* DSM 20021^T LTA [51% C$_{18:1}$(n-9) and 27% C$_{16:0}$] (18). Lactobacilli often require C$_{18:1}$(n-9) as a growth factor; this is true for *L. gasseri* JCM 1131^T (24). The Tween 80 in MRS broth serves as a source of C$_{18:1}$(n-9). Thus, *L. gasseri* JCM 1131^T is enriched with C$_{18:1}$(n-9) in its glycolipid anchor to meet the requirements of cell physiology. The incorporation of Tween 80-derived C$_{18:1}$(n-9) in the cell membrane of lactobacilli has been reported previously (25), suggesting that the fatty acid composition of the glycolipid anchor in LTA is influenced by the fatty acid composition of the culture medium.

Conclusion. The overall chemical structure of LTA from *L. gasseri* JCM 1131^T is illustrated in Fig. 2. The LTA is composed of a poly-GroP backbone. Approximately 30% of the C-2 hydroxyl groups in the GroP residues are substituted by D-alanine without substitution by a hexose. The glycolipid anchor is identified as Hex$_4$DAG and acylHex$_4$DAG, having galactose and glucose as the anchor sugars at an approximate molar ratio of 3:1. The anchor lipid contains predominantly C$_{18:1}$(n-9) in

addition to C$_{16:0}$ and C$_{18:0}$ as minor components. The novel Hex$_4$DAG and acylHex$_4$DAG structures found in the glycolipid anchor of LTA suggest that *L. gasseri* JCM 1131^T engages in a different type of host-microbe interaction from those of other gut microbes, especially in terms of modulation of the host immune system; however, the exact mechanism remains to be elucidated.

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