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# STRUCTURE OF THE LIPOTEICHOIC ACIDS FROM *BIFIDOBACTERIUM BIFIDUM* SPP. PENNSYLVANICUM

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**The lipoteichoic acids from *Bifidobacterium bifidum* spp. pennsylvanicum were extracted from cytoplasmic membranes or from disintegrated bacteria with aqueous phenol and purified by gel chromatography. The lipoteichoic acid preparations contained phosphate, glycerol, galactose, glucose and fatty acids in a molar ratio of 1.0:1.0:1.3:1.2:0.3. Chemical analysis and NMR studies of the native preparations and of products from various acid and alkaline hydrolysis procedures gave evidence for the structure of two lipoteichoic acids. The lipid anchor appeared to be 3-*O*-(6′-(*sn*-glycero-1-phosphoryl)diacyl-β-D-galactofuranosyl)-*sn*-1,2-diacylglycerol. The polar part showed two structural features not previously described for lipoteichoic acids. A 1,2- (instead of the usual 1,3-) phosphodiester-linked *sn*-glycerol phosphate chain is only used substituted at the terminal glycerol unit with a linear polysaccharide, containing either β(1 → 5)-linked D-galactofuranosyl groups or β(1 → 6)-linked D-glucopyranosyl groups.**

## Introduction

Lipoteichoic acids are membrane-associated amphiphilic molecules found in many Gram-positive bacteria [1–4]. They are structurally uniform and usually consist of a poly(1,3)glycerol phosphate backbone, in some cases substituted by alanyl ester or glycosidic groups, covalently linked to a glyco- or phosphoglycolipid [3] which is identical or closely related to one of the membrane lipids [5]. A poly(glycosylglycerol phosphate) chain was observed as a unit of lipoteichoic acid in *Streptococcus lactis* Kiel 42172 [6].

In previous studies of the cell envelope of *Bifidobacterium bifidum* spp. pennsylvanicum we elucidated the structure of the galactosyldiacylglycerols, phospholipids and phosphogalactolipids present in the membrane [7,8] and of the peptidoglycan [9], a glucosylated rhamnose polymer and a mannitol teichoic acid [10] present in the cell wall. The occurrence of glycerol, galactose and phosphate together with fatty acids in a fraction of the cell wall which was not solubilized with lysozyme suggested the presence of a lipoteichoic acid [10]. The unusually high content of glycerophosphogalactosyldiacylglycerol in the membrane of *B. bifidum* [8,11] and the parallel occurrence of glycerophosphoglycolipids and lipoteichoic acids in many Gram-positive bacteria [12] initiated our studies on the lipoteichoic acids of *B. bifidum*. The present paper describes their isolation and structural analysis.

Indications were obtained for the presence of a diacyl derivative of glycerophosphogalactosyldiacylglycerol as the lipid anchor. As structural units of the polar chain we identified a poly(1,2)-glycerol phosphate backbone and two terminal polysaccharides. The latter consisted either of glu-

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cose or of galactose. The presence of a 1,2-phosphodiester-linked glycerol phosphate backbone together with terminal polysaccharide units distinguishes the lipoteichoic acids from *B. bifidum* from all lipoteichoic acid structures described up to now.

## Materials and Methods

*Materials.* Enzymes were purchased from Boehringer (Mannheim, F.R.G.) except alkaline phosphatase (EC 3.1.3.1), which was obtained from Sigma (St. Louis, MO, U.S.A.). Sepharose 6B and DEAE-Sephacel were purchased from Pharmacia (Piscataway, NJ, U.S.A.); [1-$^{14}$C]oleic acid and $Na_2H^{32}PO_4$ from Amersham International (Amersham, U.K.). All other chemicals were of analytical grade.

*Growth of bacteria. B. bifidum* spp. pennsylvanicum was grown in a chemically defined medium containing 2% (v/v) skimmed human milk [13] under an $N_2/CO_2$ (90/10%) atmosphere for 16 h at 37 °C. When $^{32}P_i$ was used for labeling (1.0 mCi/l) the $K_2HPO_4$ concentration of the medium was lowered from 2.5 to 0.25 g/l. When [1-$^{14}$C]oleic acid (250 $\mu$Ci/l) was included in the medium the Tween-80 concentration was decreased from 0.5 to 0.1 g/l. The medium modifications did not affect growth.

*Isolation and purification of the lipoteichoic acids.* Bacteria were harvested in the early stationary phase in a water-cooled Sharples continuous-flow centrifuge. The yield was about 6 g wet weight cells/l. Cells were washed twice with 0.1 M phosphate buffer (pH 6.8) containing 5 mM $MgSO_4$ and suspended in the same buffer containing 0.7 M sucrose and 5 mM $MgSO_4$ (1 g wet weight cells/5 ml). Lysozyme (EC 3.2.1.17) was added (1 mg/g wet weight cells) and the cells were incubated at 37 °C with shaking. Protoplast formation was followed by microscope (Leitz-Wetzlar Dialux phase contrast) and was complete after 30–60 min. The protoplasts were sedimented by centrifugation at $8000 \times g$ for 20 min and sonicated in 0.1 M acetate buffer (pH 6.0) with a Branson Sonifier 70W (four times for 5 s at max. output with intervenient cooling. DNAase (EC 3.1.4.5) was added (0.1 mg/g wet weight cells) and the mixture was incubated for 10 min at 37 °C. Membranes were sedimented by ultracentrifugation ($100\,000 \times g$ for 45 min), washed twice with 5 mM $MgSO_4$ and lyophilized. Membranes were suspended in 0.1 M acetate buffer (pH 6.0) and an equal volume of 88% phenol (w/v) was added. The mixture was stirred at 4 °C for 16 h and centrifuged ($10\,000 \times g$ for 30 min). The aqueous layer was collected. The organic layer and the insoluble residue were washed with an equal volume of the acetate buffer and centrifuged as described above. The aqueous layers were combined, dialyzed against several changes of water and lyophilized. Crude phenol extract, obtained from 60 g cells (wet weight) was suspended in 30 ml of 0.1 M acetate buffer (pH 6.0) containing 5 mM $MgSO_4$. DNAase (5 mg), RNAase (EC 2.7.7.16) (20 mg) and $\alpha$-amylase (EC 3.2.1.1) (1 mg) were added and the mixture was incubated at 20 °C for 24 h. Subsequently, the mixture was extracted with 2 vol. of chloroform/methanol (1 : 1, v/v). The water layer was collected, dialyzed and, after removal of insoluble material by centrifugation, lyophilized.

The crude lipoteichoic acid material was dissolved in 0.2 M ammonium acetate (pH 7.0) and loaded on a Sepharose 6B column ($1.7 \times 50$ cm). The column was eluted with 0.2 M ammonium acetate (pH 7.0) containing 0.02% $NaN_3$. Fractions of 5.5 ml were collected. $^{32}$P- and [$^{14}$C]oleic acid-labeled lipoteichoic acids were isolated using the same procedure. For comparison, lipoteichoic acid was isolated and purified according to the recently described procedure of Fischer et al. [14] with the modification that we used sonication (15 times for 45 s with intervenient cooling) to disrupt the bacteria. Purification of the crude extract was achieved by ion-exchange chromatography on DEAE-Sephacel ($1.7 \times 45$ cm) followed by Sepharose 6B chromatography.

*Deacylation of the lipoteichoic acid.* Lipoteichoic acid was deacylated in 0.1 M NaOH in ethanol for 30 min at 37 °C. After incubation the mixture was passed through a Dowex-50 $H^+$ column and dried under reduced pressure. The deacylated lipoteichoic acid was recovered by extraction of the residue with a mixture of 1 ml water and 2 ml chloroform/isopropanol (2 : 1, v/v). The water layer was concentrated and chromatographed on Sepharose 6B or Sephadex G-75 ($1.7 \times 100$ cm,

fractions 6.0 ml). The columns were eluted with 0.2 M ammonium acetate (pH 7.0).

*Acid hydrolysis of lipoteichoic acid.* Lipoteichoic acid (1–2 mg) was hydrolyzed in 0.5 ml 2 M HCl for 2.5 h at 100 °C. For enzymatic analysis the hydrolysate was neutralized with NaOH. For GLC analysis HCl was evaporated in vacuo by several additions of water. The products were dephosphorylated with 0.5 U alkaline phosphatase for 3 h at 37 °C in 0.5 ml water, brought to pH 9.0 with diluted ammonia, and converted into alditol acetates as described before [10]. For GLC, deoxyglucose was used as internal standard. $^{32}$P-labeled lipoteichoic acid (100 000 dpm) was hydrolyzed in the same way. The HCl was evaporated and $^{32}$P-labeled compounds were analyzed by paper electrophoresis.

*Alkaline hydrolysis of lipoteichoic acid.* Unlabeled (5–25 mg) or $^{32}$P-labeled lipoteichoic acid (100 000 dpm) was dissolved in 1 ml of 1 M NaOH and heated for 1 h at 100 °C. The hydrolysates were passed through a Dowex-50 $H^+$ column to remove $Na^+$. $^{32}$P-labeled compounds were analyzed by paper electrophoresis. The hydrolysate derived from unlabeled lipoteichoic acid was applied to a Bio-Gel P2 column (2.0 × 80 cm) and eluted with water. Fractions of 3.0 ml were collected. Mild alkaline treatment was performed in 1 M NaOH for 24 h at 37 °C.

*Treatment with HF.* Unlabeled (5–25 mg) or $^{32}$P-labeled lipoteichoic acid (100 000 dpm) was hydrolyzed in 48% (w/w) HF (1 ml) at 4 °C for the periods indicated in Results. After freezing, HF was removed in vacuo over NaOH pellets. Lipid and polar compounds were separated by extraction of the residue with chloroform/methanol/water (1 : 1 : 0.9, by vol.). The lipid fraction was analyzed by thin-layer chromatography in systems A and B. The extent of hydrolysis was determined from the ratio of inorganic phosphate to total phosphate in the aqueous layer. The sugar content of the aqueous layer was determined before and after HCl hydrolysis. The aqueous layer was also concentrated and separated on a Bio-Gel P2 column by elution with water.

*Periodate oxidation.* Smith degradation of lipoteichoic acid was performed in 50 mM $NaIO_4$/0.1 M sodium acetate (pH 6.0) for 24 h at 22 °C in the dark. The oxidation was terminated with ethylene glycol. After reduction with $KBH_4$, hydrolysis with HCl or NaOH and another reduction, phosphate-containing compounds were analyzed by paper electrophoresis at pH 3.5. In another experiment the oxidation products were reduced with $KBH_4$, hydrolyzed with 2 M HCl (2.5 h at 100 °C), dephosphorylated, reduced, converted to alditol acetates and analyzed by gas chromatography [10].

Mild periodate oxidation was performed in 5 mM $NaIO_4$–0.1 M sodium-acetate (pH 6.0) for 15 min at 22 °C. The products were analyzed after reduction, mild acid hydrolysis (0.2 M HCl, 1 h, 100 °C) and further reduction as their alditol acetates [10].

Hydrazinolysis was carried out as described by Fischer et al. [15].

*Enzymatic hydrolysis procedures.* Native lipoteichoic acid and water-soluble products derived from HF treatment were incubated with 5 U of glycosidases in appropriate buffers at 37 °C. The following buffers were used: 0.1 M acetate buffer (pH 6.0) containing 2.7 mM EDTA for α-glucosidase (EC 3.2.1.20); 0.1 M acetate buffer (pH 5.0) for β-glucosidase (EC 3.2.1.21); 0.1 M phosphate buffer (pH 6.5) for α-galactosidase (EC 3.2.1.22) and 0.1 m phosphate buffer (pH 7.0) containing 2 mM $MgSO_4$ for β-galactosidase (EC 3.2.1.23). Liberated sugars and glycerol were determined enzymatically. The lipoteichoic acid and the product after periodate-oxidation and hydrazinolysis were also treated with a phosphodiesterase-phosphomonoesterase preparation from *Aspergillus niger* [16], according to the procedure of Fischer et al. [17].

*NMR study of deacylated and alkaline-hydrolyzed lipoteichoic acid.* Lipoteichoic acid-derived samples for NMR investigations were exchanged against $^2H_2O$ by 5-fold lyophilization, using 99.96 atom% deuterated water (Aldrich, Milwaukee, WI, U.S.A.) in the final step. For $^{13}$C-NMR measurements, 25 mg lipoteichoic acid-derived material were used, while for $^1$H-NMR spectroscopy 1 mg was ample. Acetone served as an internal standard. $^{13}$C chemical shifts were measured relative to the methyl carbon singlet of acetone (δ 31.40, relative to external tetramethylsilane at 25 °C), with an estimated precision of 0.05 ppm. $^1$H chemical shifts were measured relative to the methyl

proton singlet of acetone ($\delta$ 2.225, relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate at 27 °C), with an accuracy of 0.002 ppm.

The $^{13}$C-NMR spectra were recorded on a Bruker WM-200 WB spectrometer (SON hf-NMR facility, University of Nijmegen, The Netherlands), operating at 50.3 MHz under control of an Aspect 2000 computer. 10-mm sample tubes containing 1.4 ml $^2H_2O$ were used ($p^2H = 7$). The applied pulse width was 15 $\mu$s, corresponding to a flip angle of about 75 °. The spectral width was 10 000 Hz. The probe temperature was 25 °C. To avoid line-broadening due to temperature fluctuations as much as possible, a minimal proton-decoupling power (0.5 W) was employed during aquisition. During the 1.5 s relaxation delay, a lower decoupling level (0.1 W) was applied in order to maintain the Nuclear Overhauser Enhancement. For additional $^{31}$P-decoupling, a continuous broadband decoupling level of 0.4 W was applied. Spectra were recorded on 8K data points; free induction decays were multiplied by an exponential window and zero-filled to 16K data points. To determine the $^{13}$C-$^{31}$P coupling constants more accurately, $^{13}$C-NMR spectra were also recorded on a Bruker HX-360 spectrometer (SON hf-NMR facility, University of Groningen, The Netherlands), operating at 90.5 MHz under control of an Aspect 2000 computer. Sample tubes: 5 mm; solvent 0.4 ml $^2H_2O$; pulse width: 30 $\mu$s (about 90 °); spectral width: 7500 Hz; $p^2H = 7$.

The $^1$H-NMR spectra were recorded at 500 MHz with a Bruker WM-500 spectrometer (SON hf-NMR facility, University of Nijmegen), operating under control of an Aspect 2000 computer. The pulse width was about 12 $\mu$s, corresponding to a flip angle of 90 °. The spectral width was 3000 Hz. Probe temperature was 27 °C. Spectra were recorded on 16K data points. Resolution enhancement was achieved by Lorentzian-to-Gaussian transformation followed by zero-filling to 32K data points.

*Analytical assays.* Total hexose was determined by the method of Spiro et al. [18]; phosphate by the method of Bartlett [19]; protein according to Lowry et al. [20] with bovine serum albumin as standard; glucose with a Glucostat test set (Boehringer); galactose with galactose dehydrogenase (EC 1.1.1.48) [21]; glycerol with an enzymatic assay (Boehringer); formaldehyde according to Nash [22] with serine as standard and glycerol 3-phosphate with glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) as described before [8]. Labeled compounds were located after chromatography or electrophoresis by autoradiography using Kodak X-ray films ($RP/P_2$). Radioactivity was determined by liquid scintillation counting in Opti-Fluor (Packard). Amino acid analysis was performed after hydrolysis of samples in 6 M HCl for 22 h at 100 °C in vacuo with norleucine as internal standard.

Methylation of native lipoteichoic acid and of preparations after treatment with NaOH or HF and analysis of methylated hexoses was performed according to Jansson et al. [23] with some modifications [10].

Chromium trioxide oxidation was performed according to Pazur et al. [24].

For fatty acid analysis, lipoteichoic acid (1 mg) was methanolyzed in 0.5 ml hexane with 1 ml 14% $BF_3$ in methanol (w/v) for 10 min at 100 °C. After cooling, 2 ml water were added. The fatty acid methyl esters were extracted twice with 4 ml pentane. Gas chromatographic analysis was performed on a Packard 428 gas chromatograph, equipped with a 15% diethyleneglycol succinate (on Gaschrom P, 60–80 mesh) column, at 167 °C. Nonadecanoic acid was used as internal standard.

*Electrophoresis and chromatography.* Electrophoresis was carried out on Whatman 3 MM paper in acetic acid/pyridine/water (10 : 1 : 89, by vol., pH 3.5) for 2.5 h (40 V/cm, 4 °C). For quantitative analysis, paper was pretreated with 1% acetic acid before use. Spots were eluted with water. Thin-layer chromatography was performed on Silicagel 60 (Merck) in solvent system (A) chloroform/methanol/7 M ammonia (70 : 20 : 2, by vol.), (B) chloroform/methanol/acetic acid/water (125 : 37 : 9.5 : 1.5, by vol.), (C) propanol/pyridine/water (7 : 4 : 3, by vol.).

## Results

### *Isolation and purification*

Lipoteichoic acids were isolated and purified by two different procedures. In the usual procedure, protoplast formation was included to avoid contamination with cell-wall mannitol teichoic acid

and α-amylase was used to split contaminating glycogen [10]. Sepharose 6B column chromatography of the crude phenol extract is shown in Fig. 1A. The lipoteichoic acid eluted near the void volume. After rechromatography of the lipoteichoic acid-containing fractions (Fig. 1B), the resulting purified lipoteichoic acid preparation had a high hexose content and contained negligible amounts of nucleic acids (absorbance at 260 nm). The high hexose content is not due to a contamination of the preparation with free cell-wall polysaccharides, since the carbohydrate and phosphate elution profiles from the gel permeation chromatography were coincident and symmetrical. The yield was about 85 mg lipoteichoic acid/100 g cells (wet weight).

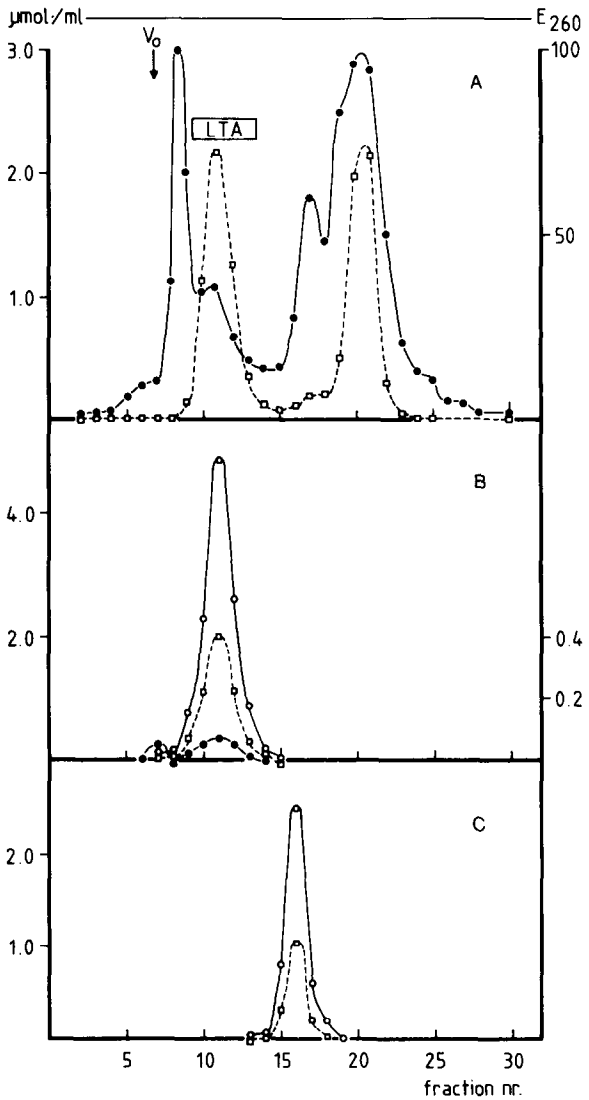


Fig. 1. Sepharose 6B chromatography of lipoteichoic acid and its deacylation product. Load; 100 µmol phosphate. Elution was performed with 0.2 M ammonium acetate (pH 7.0) containing 0.02% $NaN_3$. Fractions of 5.5 ml were collected and assayed for phosphate (□), hexose (○) and nucleic acid material (●, $E_{260}$). (A) Crude phenol extract; (B) rechromatography of lipoteichoic acid-containing fractions from A; (C) lipoteichoic acid after mild alkaline treatment (deacylation).

For comparison we also used the method recently described by Fischer and coworkers [14]. Phenol extraction was performed on disrupted cells without any pretreatment. The crude lipoteichoic acid preparation was applied on DEAE-Sephacel. The elution profile is shown in Fig. 2. The lipoteichoic acid (peak II) was well separated from contaminating polysaccharide (peak I) and nucleic acid (peak III). Although the lipoteichoic acid peak was rather broad, suggesting a heterogeneous composition, chromatography of this peak on Sepharose 6B gave exactly the same elution profile as the first procedure (Fig. 1B). The yield of the Fischer procedure was about 220 mg/100 g cells (wet weight). For both procedures, free lipids, included in the micelles, could be removed by extraction of the pure lyophilized lipoteichoic acid preparations with chloroform/methanol (2 : 1, v/v)

We also tried to purify the lipoteichoic acid using Octyl-Sepharose [14]. This purification step was not useful, since only a minor amount of the lipoteichoic acid bound to the column, probably due to the high hexose content.

The lipoteichoic acid did not absorb to phoshatidylcholine vesicles as was described for intra- and extracellular lipoteichoic acids from *Streptococcus mutans* [25], so this technique could not be used for purification of the bifidobacterial lipoteichoic acid.

*Deacylation of the lipoteichoic acid preparation*

Mild alkaline hydrolysis resulted in deacylation of the lipoteichoic acid. The deacylated lipoteichoic acid eluted on Sepharose 6B near the inner volume (Fig. 1C), indicating a micellar composition of the native material. Elution of the deacylated molecule on Sepharose G-75 indicated a molecular weight of about 10000. The deacyl-

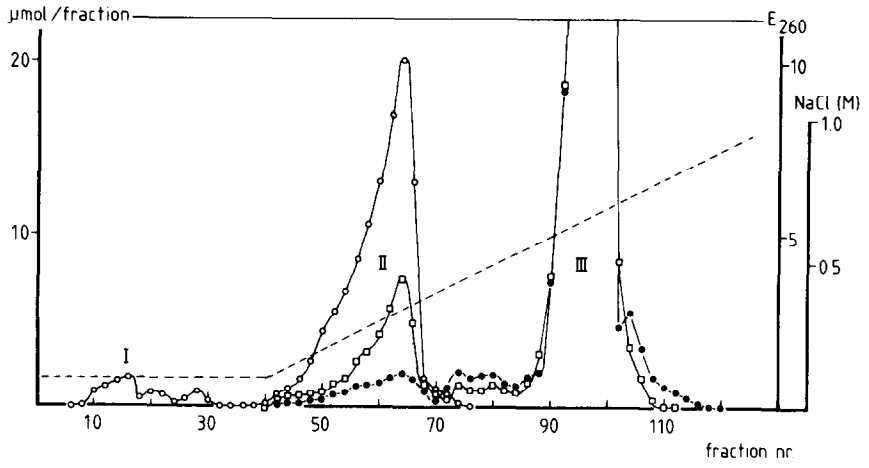


Fig. 2. Chromatography of crude phenol extract from disintegrated bacteria on DEAE-Sephacel [14]. Load; 250 µmol phosphate. Elution was performed with 0.1 M sodium acetate (pH 4.7) containing 0.05% Triton X-100 followed by a gradient of 0–1 M NaCl in the same buffer (- - - -). Fractions of 6.0 ml were collected and assayed for phosphate (□), hexose (○) and nucleic acid material (●, $E_{260}$).

ated product, however, eluted as a rather broad peak.

*Chemical analysis*

The chemical composition of the purified lipoteichoic acid preparations and the deacylation product is given in Table I. The two purification procedures gave comparable results. Glycerol, galactose and glucose were identified and quantitated as their alditol acetates or as their trimethyl silyl ethers by gas chromatography. Analysis was also performed by enzymatic assay. The latter assays proved that galactose and glucose possess the D-configuration. The galactose/glucose ratio was $0.94 \pm 0.28$, indicating a rather great divergence between different preparations. Glycerol and phosphate are present in equimolar amounts. No ribitol was detected. This showed the absence of nucleic acid material in the lipoteichoic acid preparations and excluded the presence of ribitol lipoteichoic acid [26].

The fatty acid composition is given in Table II. The major fatty acids are palmitic and octadecenoic acid. The protein content of the purified lipoteichoic acid preparations, determined as Lowry-positive material or by amino acid analysis, varied from 1.3 to 4.8%. Amino acid analysis showed only the presence of a slight amount of the peptidoglycan amino acids [9] with alanine in a proportional amount. This excludes the presence of alanyl ester substituents in the lipoteichoic acid. No other constituents of the cell wall (rhamnose, mannitol) were observed.

TABLE I

CHEMICAL COMPOSITION OF THE LIPOTEICHOIC ACID AND ITS DEACYLATION PRODUCT

Values are means ± S.D. of seven and five separate preparations of lipoteichoic acid and deacylated lipoteichoic acid, respectively. LTA, lipoteichoic acid; dLTA, deacylated lipoteichoic acid.

| Component | LTA (µmol/mg) | Molar ratio to phosphate (LTA) | dLTA (µmol/mg) |
|---|---|---|---|
| Galactose | 1.33 ± 0.19 | 1.29 ± 0.29 | 1.55 ± 0.21 |
| Glucose | 1.24 ± 0.38 | 1.20 ± 0.39 | 1.29 ± 0.39 |
| Glycerol | 1.03 ± 0.11 | 1.00 ± 0.06 | 1.28 ± 0.15 |
| Phosphate | 1.05 ± 0.14 | 1.00 | 1.25 ± 0.11 |
| Fatty acids | 0.31 ± 0.08 | 0.30 ± 0.05 | – |

TABLE II

FATTY ACID COMPOSITION OF THE LIPOTEICHOIC ACID

Values (mean ± S.D. of five preparations) are given as mol%.

| Fatty acid | % of total fatty acids |
|---|---|
| 12:0 | 1.9 ± 0.8 |
| 14:0 | 9.3 ± 1.0 |
| 15: anteiso | 1.2 ± 0.9 |
| 16:0 | 31.9 ± 3.0 |
| 16:1 | 4.2 ± 0.4 |
| 18:0 | 9.6 ± 1.2 |
| 18:1 | 39.8 ± 2.6 |
| 19:0 cyclo | 2.7 ± 0.6 |

*Structural analysis*

On HCl hydrolysis of $^{32}$P-labeled lipoteichoic acid about 68% of the phosphate was released as glycerol monophosphate, 7% as inorganic phosphate and 12% as hexose phosphate, as determined by paper electrophoresis. The hexose phosphate could be identified as galactose monophosphate. HCl hydrolysis of unlabeled lipoteichoic acid liberated glycerol (23% of total amount), galactose (85% of total amount) and glucose (100% of total amount). Phosphatase treatment released additional glycerol and some galactose. Strong alkaline hydrolysis (1 M NaOH, 1 h, 100 °C) of $^{32}$P-labeled lipoteichoic acid resulted in the release of 95% of total phosphate as glycerol monophosphate. The resulting 5% appeared as a rather diffuse spot with $M_{Pi}$ 0.20–0.40. With milder hydrolysis conditions these products accounted for 81 and 19%, respectively. Enzymatic analysis, after acid hydrolysis and dephosphorylation of the rather immobile compound, showed the presence of a small amount of glycerol together with almost all of the galactose and glucose. No glycerol diphosphate, diglycerol phosphate or phosphoryl-glyceryl-phosphoryl-glycerol phosphate were detected in the acid nor in the alkaline hydrolysate.

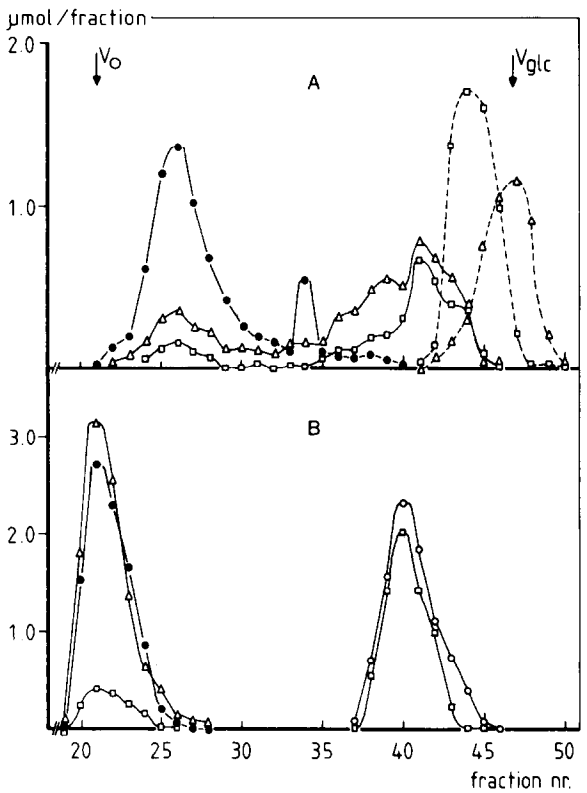


Fig. 3. Chromatography of HF (A) and alkaline (B) hydrolysates from lipoteichoic acid on Bio-Gel P2. Elution was performed with water. Fractions of 3.0 ml were collected and assayed for phosphate (○), galactose (△), glucose (●) and glycerol (□). Dotted lines indicate free sugars, solid lines bound components.

In another experiment we applied the strong alkaline hydrolysate from lipoteichoic acid on a Bio-Gel P2 column (Fig. 3B). The peak eluting in the void volume contained galactose/glucose/glycerol in a ratio of 1.0 : 1.0 : 0.1. Almost the total amount of galactose and glucose present in the lipoteichoic acid preparation eluted in this peak. The second peak eluting near the inner volume could be identified as being glycerol monophosphate together with a small amount of galactosylglycerol. No free galactose, glucose or glycerol was detected.

We also studied the configuration of the glycerol monophosphates obtained from acid and alkaline hydrolysis. With stereospecific glycerol-3-phosphate dehydrogenase we determined the amount of glycerol 3-phosphate after alkaline hydrolysis [8]. Glycerol 2-phosphate was determined by use of periodate oxidation followed by reduction and dephosphorylation. The resulting glycerol must originate from glycerol 2-phosphate. The value for glycerol 1-phosphate can be derived from the former results. 39, 1 and 60% of the glycerol phosphate released by alkaline hydrolysis appeared to be present as glycerol 1-phosphate,

TABLE III

LIBERATION OF WATER-SOLUBLE LIPOTEICHOIC ACID COMPONENTS DURING TREATMENT WITH HF

Values are means ± S.D. of three to seven separate experiments and expressed in percents of the total amount present. n.d., not detected.

| Component | Hydrolysis time | | |
|---|---|---|---|
| | 48 h | 96 h | 240 h |
| Galactose | 32 ± 6 | 38 ± 5 | 73 ± 4 |
| Glucose | 2 ± 1 | 7 ± 5 | n.d. |
| Glycerol | 51 ± 5 | 64 ± 9 | 75 ± 3 |
| Phosphate | 63 ± 9 | 70 ± 8 | 79 ± 7 |

glycerol 3-phosphate and glycerol 2-phosphate, respectively. After acid hydrolysis, 63 and 37% of glycerol phosphate was present as α- and β-isomer, respectively. Since no diglycerol phosphate, glycerol diphosphate or phosphoryl-glyceryl-phosphoryl-glycerol phosphate was observed after acid or alkaline hydrolysis, but only glycerol monophosphate, a 1,3-phosphodiester-linked glycerol phosphate chain is unlikely [27]. Alkaline treatment does not cause phosphate migration [28,29]. Therefore, the released α-isomer retains the stereochemical configuration that it had in the parent compound. The presence of glycerol 1-phosphate in the alkaline hydrolysat, together with the $^{13}C$-NMR data (see below), gave evidence for poly(1,2)glycerol phosphate being a structural unit of the lipoteichoic acid molecule. The high alkaline lability of the glycerol phosphate chain further indicates that C-3 of glycerol is not substituted with glycosidic groups.

*Treatment with HF*

Table III shows the influence of time on liberation of water-soluble lipoteichoic acid components during HF hydrolysis. Glucose is liberated only to a slight extent by HF treatment. Considerable amounts of galactose and glycerol were liberated, which suggests that these components are both present as phosphate esters in the lipoteichoic acid. This is not consistent with the results from the alkaline hydrolysis, since galactose remains bound after this treatment. Free galactose increases after longer hydrolysis periods with HF, resulting in the liberation of 73% of total galactose after 240 h. HF obviously splits not only phosphomono- and diesters in this case, but also glycosidically bound galactose. This suggests the presence of galactofuranosyl groups, since this glycosidic linkage is very acid labile. With the deacylated lipoteichoic acid the same results were obtained.

The elution profile of an HF-hydrolysate (96 h) loaded on Bio-Gel P2 (Fig. 3A) is completely different from that of an alkaline hydrolysate (Fig. 3B). In the former the galactose is spread over the whole column volume, indicating the partial degradation of a galactan into several fragments. The glucose remains bound in a large compound, since it eluted near the void volume.

*Enzymatic hydrolysis*

Incubation of the lipoteichoic acid preparation or the products from alkaline hydrolysis with glycosidases did not liberate glucose or galactose. This supports the presence of these two sugars in a rather large polysaccharide and not as mono- or oligosaccharide substituents on the glycerol phosphate backbone. Treatment of the water-soluble products after HF hydrolysis (96 h) with galactosidases resulted in the additional liberation of about 15% of total galactose together with some glycerol (5%) after 24 h. α-Glucosidase had no effect. β-Glucosidase liberated 10 and 20% of glucose after 24 and 112 h, respectively.

Incubation of the lipoteichoic acid with alkaline phosphatase resulted in the liberation of 0.5% of total phosphate as free phosphate, indicating the absence of phosphate monoesters. Treatment of the lipoteichoic acid with the phospodiesterase-phosphomonoesterase preparation of *Aspergillus niger* [16] resulted in the liberation of only 12% of total glycerol after 24 h. Degradation of the polysaccharide part of the lipoteichoic acid by periodate oxidation and hydrazinolysis, however, allowed the liberation of 71% of total glycerol within 24 h. The phosphodiesterase removes the terminal glycerol from a poly(glycerol phosphate) chain, leaving a phosphomonoester which in turn is hydrolyzed by the phosphomonoesterase [16,17]. From the former results we may conclude that most, if not all, terminal glycerols of the poly(glycerol phosphate) chains in the lipoteichoic acids are linked to polysaccharides containing galactose and/or glucose. Furthermore, after degradation of the lipoteichoic acid by periodate oxidation, hydrazinolysis and phosphodiesterase-phosphomonoesterase treatment, we could detect a small amount of galactosylglycerol, originating from the lipid anchor, by thin-layer chromatography on Silicagel 60 in system C.

*Methylation analysis*

In order to define the types of the glycosidic bonds in which galactose and glucose are involved in the lipoteichoic acid molecule, we carried out methylation analysis on native lipoteichoic acid and its deacylation product as well as on alkaline and HF (96 h) hydrolysates. The results are compiled in Table IV. The native lipoteichoic acid

TABLE IV

PERMETHYLATION ANALYSIS OF NATIVE AND DEACYLATED LIPOTEICHOIC ACID AND OF PREPARATIONS OBTAINED AFTER TREATMENT WITH NaOH OR HF

Values (mean ± S.D. of three separate experiments) are peak area ratios relative to 2,3,4-trimethylglucitol. LTA, lipoteichoic acid, dLTA, deacylated lipoteichoic acid.

| Methylated alditol acetate | LTA | dLTA | LTA/NaOH | LTA/HF |
|---|---|---|---|---|
| 2,3,4-Trimethylglucitol | 1.00 | 1.00 | 1.00 | 1.00 |
| 2,3,4,6-Tetramethylglucitol | + [a] | + | + | 0.28 ± 0.14 |
| 2,3,6-Trimethylgalactitol | 0.80 ± 0.08 | 0.75 ± 0.10 | 0.83 ± 0.10 | – |
| 2,3,5-Trimethylgalactitol | + | + | + | 0.20 ± 0.05 |
| 2,3,4,6-Tetramethylgalactitol | – [b] | – | – | 0.70 ± 0.30 |
| 2,3,5,6-Tetramethylgalactitol | + | + | + | 0.70 ± 0.25 |

[a] < 0.1.

[b] not detectable ( < 0.01).

shows the presence of two major methylated alditol acetates, namely 2,3,4-trimethylglucitol, indicating the presence of C-6-substituted glucopyranosyl residues, and 2,3,6-trimethylgalactitol, derived from C-5-substituted galactofuranosyl groups. Besides these two main constituents we could also detect 2,3,4,6-tetramethylglucitol originating from terminal glucopyranosyl groups; 2,3,5,6-tetramethylgalactitol from terminal galactofuranosyl groups and 2,3,5-trimethylgalactitol from C-6-substituted galactofuranosyl residues. Methylation analysis of the deacylation product and the alkaline hydrolysate showed the same products as described above, in identical ratios. This confirms that, under the alkaline hydrolysis conditions used, glycosidic bonds are not split. Analysis of the HF-hydrolysate shows the absence of C-5-substituted galactofuranosyl groups and an increase in the amount of unsubstituted, terminal galactofuranosyl residues. In addition, we detected a new peak, namely 2,3,4,6-tetramethyl galactitol, originating from unsubstituted galactopyranosyl residues which were formed after splitting of some furanosyl linkages by HF.

TABLE V

$^{13}C$ CHEMICAL SHIFTS OF DEACYLATED LIPOTEICHOIC ACID, ITS ALKALINE HYDROLYSATE AND RELEVANT REFERENCE SUBSTANCES

Chemical shifts are expressed in ppm downfield from external tetramethylsilane (measured relative to internal acetone, at $\delta$ 31.40, in $^2H_2O$ at 25 °C). $^{31}P$-$^{13}C$ coupling constants (in parentheses) are in Hz. Teichoic acid consists of a 2,3-phosphodiester-linked glycerol phosphate chain, substituted with glucose at C-1 of glycerol. LTA, lipoteichoic acid, dLTA, deacylated lipoteichoic acid.

| Compound | Chemical shifts of | | | | | | | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| | C–1′ | C–2′ | C–3′ | C–4′ | C–5′ | C–6′ | C–1 | C–2 | C–3 | |
| Glycerol | – | – | – | – | – | – | 63.8 | 73.3 | 63.8 | [32] |
| Glycerol 2-phosphate | – | – | – | – | – | – | 62.5(3.8) | 75.1(5.0) | 62.5(3.8) | [32] |
| Glycerol 3-phosphate | – | – | – | – | – | – | 62.8 | 71.6(6.4) | 65.2(4.8) | [32] |
| Poly(1,3)glycerol phosphate | – | – | – | – | – | – | 66.3(5.6) | 69.8(8.1) | 66.3(5.6) | [33] |
| Teichoic acid | 103.3 | 73.6 | 76.1 | 70.1 | 76.3 | 61.2 | 69.5(4.0) | 75.3 [a] | 66.1 [a] | [32] |
| Methyl β-glucopyranoside | 104.0 | 74.1 | 76.8 | 70.6 | 76.8 | 61.8 | – | – | – | [34] |
| Methyl β-galactofuranoside | 109.2 | 81.9 | 77.8 | 84.0 | 72.0 | 63.9 | – | – | – | [35] |
| dLTA (Glc) | 104.2 | 74.2 | 76.8 | 70.7 | 76.1 | 70.0 | 67.7 [a] | 71.9(6.5) | 63.3 [a] | |
| LTA/NaOH (Glc) | 104.1 | 74.2 | 76.7 | 70.6 | 76.0 | 69.9 | – | – | – | |
| (Gal) | 108.1 | 82.4 | 77.5 | 82.4 | 76.7 | 62.2 | – | – | – | |

[a] Poorly resolved; coupling constant could not be determined.

*Periodiate and chromium trioxide oxidation*

Treatment of the lipoteichoic acid with 5 mM $NaIO_4$ followed by mild acid hydrolysis (0.1 M HCl, 90 min, 100 °C) resulted in the formation of a small amount of arabinose. This indicates the presence of terminal non-reducing galactofuranosyl groups. Treatment of the water-soluble products after HF hydrolysis (96 h) in the same way resulted in a 3-times larger amount of arabinose. This is consistent with the increase of terminal galactofuranosyl residues after HF hydrolysis shown with permethylation.

Smith degradation of native lipoteichoic acid resulted in the consumption of 3.2 μmol periodate and the production of 0.88 μmol formaldehyde per mg lipoteichoic acid. A part of the oxidized lipoteichoic acid was analyzed after HCl hydrolysis, dephosphorylation and preparation of alditol acetates. All glucose and nearly all galactose appeared to be oxidized. Gas chromatography revealed the presence of glycerol (2.10 μmol/mg), erythritol (0.27 μmol/mg) and threitol (0.22 μmol/mg). The high amount of glycerol is derived from the glycerol phosphate backbone and from the C-6-substituted glucopyranosyl groups. The threitol originated from C-5-substituted galactofuranosyl groups and erythritol may be derived from incomplete peroxidation of liberated galactofuranose and C-6-substituted glucopyranosyl residues. The high amount of formaldehyde formed also indicated the degradation of the galactan chain.

Chromium trioxide oxidation of lipoteichoic acid preparations resulted in a total oxidation of galactose and glucose. This indicates that glucose molecules are present in β-glycosidic linkages, since α-hexapyranoside bonds are not affected by chromium trioxide [24]. This gives no indication about the galactose anomeric form, since both galactofuranoside anomers are degraded under the conditions used [30].

*$^{13}C$- and $^{1}H$-NMR spectroscopic analysis*

The $^{13}C$-NMR spectrum of the pure, native lipoteichoic acid was rather complex, owing to the presence of *O*-acyl groups. The occurrence of the native molecules in micelles gave rise to broad, unresolved signals. Alteration of the amphiphilic character by deacylation resulted in a simplified $^{13}C$- and $^{1}H$-NMR spectrum; apparently, this was due to reduction of aggregation [31]. Relevant chemical shifts are compiled in Table V.

The $^{13}C$-NMR spectrum of deacylated lipoteichoic acid was dominated by six well-defined singlets (Table V) stemming from C-1′ to C-6′ of β-glucopyranosyl residues that bear a substituent monosaccharide at C-6′. The latter can be concluded from the chemical shift displacements of C-6′ (+8.2 ppm) and C-5′ (−0.7 ppm) in deacylated lipoteichoic acid as compared to methyl β-glucopyranoside. The β-anomeric configuration is supported by the H-1 signal in the $^{1}H$-NMR spectrum at δ 4.528 ($J_{1,2}$ 7.8 Hz). Three additional signals of lower intensity are observable in the $^{13}C$-NMR spectrum which were attributed to C-1, C-2 and C-3 of glycerol forming part of a 1,2-phosphodiester linked poly(glycerol phosphate) chain. Assignments were based on comparison with data for glycerol, glycerol 2-phosphate and glycerol 3-phosphate (Table V). The possibility of a 1,3-linked poly(glycerol phosphate) could be excluded because the C-1 and C-3 signals for deacylated lipoteichoic acid were found to be clearly different in chemical shift (compare poly(1,3) and substituted poly(2,3) compounds, Table V). In principle, $^{13}C$-NMR cannot distinguish between 1,2- and 2,3-linked poly(glycerol phosphate). However, from the stereochemical analysis of glycerol monophosphate liberated by alkaline hydrolysis (see above) we could rule out the occurrence of 2,3-linked poly(glycerol phosphate). The assignments of the glycerol carbon signals were proved by a $^{31}P$-decoupling experiment resulting in complete removal of the $^{2}J$ and $^{3}J(^{31}P\text{-}^{13}C)$ couplings on C-1, C-2 and C-3.

Signals from galactosyl carbons could hardly be observed in the $^{13}C$-NMR spectrum of deacylated lipoteichoic acid, although these residues are present (Table I). Apparently, these signals are broadened, most probably due to restricted molecular motion. $^{31}P$-decoupling did not affect the line width of these signals. However, the $^{13}C$-NMR spectrum of the Bio-Gel P2 void volume peak of alkaline hydrolyzed lipoteichoic acid (Fig. 3B) permitted, besides the confirmation of the presence of (· → 6)Glc *p*-β(1 → ·) units, the definition of the galactosyl ring form and linkage configuration. A second set of six singlets was observed (Table V)

and ascribed to (· → 5)Gal *f*-β(1 → ·) units. The chemical shift of the C-1′ of galactose (δ 108.1) is very typical of β-galactofuranosyl residues [36]. This is substantiated by the occurrence of a singlet for H-1 of this galactosyl residue in the $^1$H-NMR spectrum at δ 5.186. The galactofuranosyl residues are substituted at C-5′ by another sugar, as was evident [36] from the shift increment of C-5′ (+4.7 ppm) and decrements of C-4′ (−1.6 ppm) and C-6′ (−1.7 ppm), compared to methyl β-galactofuranoside (Table V). The amount of glycerol present in the void volume peak was too low to obtain further information on its linkage by NMR.

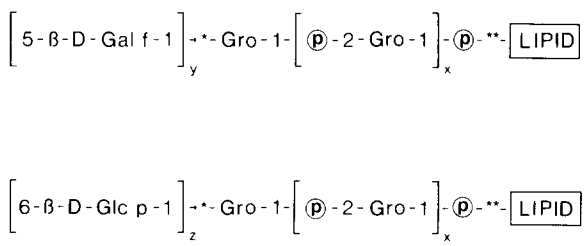


Fig. 4. Structure of the lipoteichoic acids from *B. bifidum* ssp. pennsylvanicum. Lipid = 3-*O*-(6′-(*sn*-glycero-1-phosphoryl)-diacyl-β-D-galactofuranosyl)-*sn*-1,2-diacylglycerol. ■ P indicates phosphate. * linkage either to C-2 or to C-3 of glycerol. ** linkage to C-2 of glycerol phosphate of the lipid anchor.

*The lipid part of the lipoteichoic acid*

HF is usually applied to liberate the lipid anchor from the lipoteichoic acids [6,37]. Therefore, we extracted an HF hydrolysate (96 h) from [$^{14}$C]oleic acid-labeled lipoteichoic acid with chloroform/methanol/water (1 : 1 : 0.9, by vol.). The chloroform-soluble compounds (90% of radioactivity) could be identified by thin-layer chromatography, in systems A and B, as monoacylglycerol, diacylglycerol and free fatty acid accounting for 13, 25 and 47% of total radioactivity applied, respectively. These results indicated that HF degraded the lipid anchor. Other hydrolysis conditions using chloroform/methanol/3.8 M HCl (1 : 1 : 0.3, by vol.), 0.2 M HCl [38], moist acetic acid [37] or ammonia [39] yielded the same products as described above. The results may suggest that diacylglycerol is the lipid anchor, as was found in a mutant of *Bacillus licheniformis* [40], but it appears more probable that we did not succeed in isolating the intact lipid part of the lipoteichoic acid. This may have been caused by acid degradation, since the phosphogalactolipids, the presumably lipid portion, showed the same products at HF treatment [8]. Indirect evidence for the occurrence of a phosphogalactolipid as membrane anchor came also from some other experiments. The acid hydrolysate from lipoteichoic acid contained some galactose phosphate as shown by paper electrophoresis and phosphatase treatment. These results are comparable with those from acid hydrolysis of glycerophosphorylgalactosyldiacylglycerol [8]. Analysis of the products after alkaline hydrolysis and after degradation of the lipoteichoic acid by periodate oxidation, hydrazinolysis and phosphodiesterase-phosphomonoesterase treatment revealed the presence of a small amount of galactosylglycerol identified by thin-layer chromatography on Silicagel 60 in system C. Permethylation analysis of native lipoteichoic acid revealed some 2,3,5-trimethylgalactitol originating from C-6-substituted galactofuranosyl groups. This compound was also found after permethylation of the phosphogalactolipids from *B. bifidum*. Therefore, glycerophosphorylgalactosyldiacylglycerol or one of its acyl derivatives [8] could form the lipid portion. The high content of fatty acids and the recovery of galactosylglycerol after periodate oxidation as described above indicate that a diacyl derivative is the most probable lipid anchor of the lipoteichoic acid. Such a component (lipid 6) has been isolated previously from *B. bifidum* [8].

## Discussion

The lipoteichoic acids from *Bifidobacterium bifidum* were isolated from membranes or from desintegrated bacteria by extraction with phenol. Further purification was achieved by column chromatography on Sepharose 6B and DEAE-Sephacel (Figs. 1 and 2). The procedure described by Fischer et al. [14] gives a 2.5-times higher yield than our procedure. In the latter procedure a part of the lipoteichoic acid may be lost during protoplast formation by lysozyme treatment [41,42]. The purified lipoteichoic acid preparations contained a high amount of hexoses, which were identified as galactose and glucose. No contamination with cell-wall polysaccharide or teichoic acid [10] was detected.

The structure of the lipoteichoic acids from *B. bifidum* is summarized in Fig. 4. Four structural units were identified. NMR analysis together with the analytical results revealed the presence of a poly(1,2)glycerol phosphate chain, without substitution on C-3 of glycerol, as the backbone of the lipoteichoic acid molecules. Additional evidence for this structural unit came from preliminary biosynthesis experiments. Phosphatidylglycerol appeared to be the precursor for synthesis of the poly(glycerol phosphate) chains. Since phosphatidylglycerol functions as a donor for *sn*-glycerol 1-phosphate unit, the backbone must consist of 1,2-phosphodiester linked units. HF hydrolysis, usually applied to study the glycosidic substitution [43,44], appeared to be inadequate for structural analysis because HF also split the galactofuranosyl linkages. By alkaline hydrolysis, permethylation analysis and NMR we identified two polysaccharide parts of the lipoteichoic acids, i.e., poly((1 → 5)-β-D-galactofuranose) and poly((1 → 6)-β-D-glucopyranose). The structure of a polysaccharide containing both sugars alternately is not possible because HF split the galactofuranosyl linkages, but did not liberate glucose (Table III, Fig. 3A). The polysaccharide part of the lipoteichoic acid will be linked to the terminal glycerol of the poly(glycerol phosphate) chain, since only a slight amount of glycerol was liberated during treatment with phosphodiesterase-phosphomonoesterase and the amount of glycerol released strongly increased after degradation of the polysaccharide part by periodate oxidation and hydrazinolysis. This enzyme system needs a terminal glycerol for initiation [16,17].

Due to the presence of terminal polysaccharides, the chain length of the glycerol phosphate backbone could not be determined from periodate oxidation experiments as in Ref. 43. Assuming four fatty acids per molecule lipoteichoic acid, we calculated an average chain length of 13 glycerol phosphate units linked to 32–35 hexose (galactose or glucose) molecules. There appeared to be some heterogeneity in the lipoteichoic acid preparations on account of chain length, as can be seen from DEAE-Sephacel elution of native lipoteichoic acid (Fig. 2), from the behaviour of deacylated lipoteichoic acid on Sephadex G-75 and from the large variation of the hexose to phosphate ratio.

The substitution of the terminal glycerol at C-2 or C-3 with one polysaccharide chain appears to be well in agreement with the apparent molecular weights observed at gel filtration of the deacylation product and the degradation products after alkaline hydrolysis, although the presence of two saccharide chains of about 16 hexoses at C-2 and C-3 of the terminal glycerol could not be completely excluded. The polysaccharide chain will consist either of galactose or of glucose, since methylation analysis of native lipoteichoic acid revealed the presence of terminal galactofuranosyl and glucopyranosyl groups, no heterooligosaccharide was found and a combination of two different chains of about 16 sugars does not appear to be probable from a biosynthetic viewpoint. The variation of the galactose/glucose ratio in the lipoteichoic acid preparations suggested differences in the proportion of two lipoteichoic acids. We did not succeed in separating these different molecules. Within one purification procedure all fractions tested showed the same galactose-to-glucose ratio.

The lipid moiety of lipoteichoic acids is usually a glycolipid or phosphoglycolipid normally present in the membrane [3,5]. Recently, Fischer [12] showed the relationship between the glycerophosphoglycolipids present in the membrane and lipid part of the lipoteichoic acid molecule within 33 different Gram-positive bacteria. A diacyl derivative of glycerophosphoryl galactosyldiacylglycerol, previously described [8] as lipid 6 from *B. bifidum*, appeared to be the most probable lipid anchor of the lipoteichoic acids on account of the high fatty acid content of the lipoteichoic acid preparations. The biosynthesis of this lipid by transfer of the *sn*-glycerol 1-phosphate of phosphatidylglycerol to diacylmonogalactosyldiacylglycerol [8,11] indicates that the additional acyl groups are linked to the galactose molecule and that the free glycerol unit can be used for polymerization of glycerol phosphate units during lipoteichoic acid synthesis.

So far, two types of glycerol lipoteichoic acids, in which repeating units are linked through phosphodiesters, have been described. The first and main type consists of a linear 1,3-phospodiester-linked glycerol phosphate chain variously substituted with D-alanyl ester or glycosidic groups

[1–4]. A second type shows the presence of one or more glycosides in the backbone of the molecule [6]. The lipoteichoic acids from *B. bifidum* appear to be unique, since a poly(1,2)glycerolphosphate backbone and terminal substitution of this backbone with polysaccharides have not been described before. Studies concerning biosynthesis, immunological characterization and function of the bifidobacterial lipoteichoic acids are in progress.

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