Structures of Two Monomeric Units of Teichoic Acid Prepared from the Cell Wall of Lactobacillus plantarum NRIC 1068

Satoru Tomita,1,2 Kazuo Furihata,2 Tomoo Nukada,3 Eiichi Satoh,1 Tai Uchimura,1 and Sanae Okada1

1Department of Applied Biology and Chemistry, Faculty of Applied Bio-Science, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya, Tokyo 156-8502, Japan
2Division of Agriculture and Agricultural Life Science, The University of Tokyo, 1-1-1 Yayoi, Bunkyo, Tokyo 113-8657, Japan
3Department of Fermentation Science, Faculty of Applied Bio-Science, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya, Tokyo 156-8502, Japan

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The cell wall of Lactobacillus plantarum contains large amounts of cell wall teichoic acid (WTA). WTA was isolated from the cell wall of L. plantarum NRIC 1068 (= ATCC 8014 and 17-5) by extraction with trichloroacetic acid, and two monomeric units (F1 and F2) were prepared from the alkaline hydrolysate of WTA. Componential analysis by HPLC showed that these monomers were composed of ribitol, glucose, and phosphoric acid. Structural analyses of the monomers were performed by NMR spectroscopy with comparison to chemically synthesized monomers. The structures of F1 and F2 were determined to be 3,4-α-D-diglucosyl-1-phosphoryl ribitol and 3,4-α-D-diglucosyl-1-phosphoryl ribitol respectively. The unique structure of WTA in L. plantarum results from modification of the main chain with multiple glucose residues.

Key words: teichoic acid; cell wall structure of lactic acid bacteria; Lactobacillus plantarum

The cell walls of Gram-positive bacteria consist of peptidoglycan, polysaccharide, and teichoic acid. These structures thickly cover the cell membrane and provide cell shape, and they play important roles in resistance to external mechanical, chemical, thermal, and osmotic stresses.1,2) Furthermore, the cell walls of lactic acid bacteria not only are indispensable to the life support of the bacteria, but also affect bacterial colonization of the host intestinal epithelial cells and immune stimulation of the host.3,4) It was recently made clear that the cell wall plays an important role in the beneficial effects of intestinal lactic acid bacteria on human health, for example, through intestinal and immune system regulation. These effects are often strain-dependent,5,6) but little is known about their mechanisms. The compositions of the cell walls are basically similar within a species, but sometimes differences in components are observed among strains. These differences are used to characterize the strains. Consequently, to understand the role of the cell walls of lactic acid bacteria and the structure-function relationship at the strain level, elucidation of the intraspecific diversity of the cell wall is absolutely necessary.

Lactobacillus plantarum is a lactic acid bacterium that is widely isolated from the environment and fermented foods, and is expected to be applied in the production of various fermented foods and useful materials. The cell walls of L. plantarum are composed of peptidoglycan containing meso-DAP, teichoic acid (glycerol or ribitol type); and polysaccharide composed of glucose and rhamnose.7) Although the peptidoglycan is a major component of the cell wall, its analysis is used for species identification and is not appropriate for estimating intraspecific diversity. Cell wall teichoic acid (WTA) is a second abundant component next to the peptidoglycan in the cell walls of lactic acid bacteria. It is known to have various functions8,9) and to differ in components and configuration at the strain level within the same species.10-13) Hence, we anticipated that the structural diversity of the cell wall among strains of L. plantarum could be defined by investigating WTA structure.

The chemical composition of WTA in Lactobacillus species has been examined widely for species characterization. Lactobacillus plantarum has been found to be unique, because either glycerol-type or ribitol-type WTA is contained within the same species.14) The cell wall of L. plantarum generally contains a considerable amount of WTA. The structures of glycerol- and of ribitol-type WTA were originally analyzed and estimated by chemical decomposition methods15,16) but in recent years, the structures of the WTA of Bacillus subtilis and Staphylococcus aureus have been determined by modern analytical methods. Hence, we used nuclear magnetic resonance (NMR) spectroscopy for structural determination. NMR is useful for the nondestructive analysis and comparison of spectra among samples. Additionally, we used several synthetic monomers to determine the absolute stereostructure of WTA. Here we report the structure of the monomeric units of WTA isolated from L. plantarum NRIC* 1068

* NODAI Culture Collection Center, Tokyo University of Agriculture, Tokyo, Japan

1 To whom correspondence should be addressed. Fax: +81-3-5477-2537; E-mail: ttagggtelomere@gmail.com
et al. were prepared as described by Archibald. WTA-containing fractions were collected and lyophilized. The residue was dissolved in a small amount of water and used in the preparation of monomeric units of WTA. The cells were collected by continuous centrifugation at 10,000 rpm and washed twice with 20 mM phosphate buffer (pH 7.0). The harvested cells were suspended in the same buffer and disrupted by ultrasonic disruptor (UD-201, Tomy Seiko, Tokyo) while they were cooled with iced water. After centrifugation (12,000 rpm, 4 °C, 30 min), the whitish upper layer of the pellet was collected as the disrupted fraction, and was washed with the phosphate buffer. The crude cell wall fraction was treated with 200 ml of 2% sodium dodecyl sulfate at 100 °C for 1 h, and then was washed with distilled water thoroughly. Subsequently, the fraction was treated with 200 ml of each enzyme solution, Pronase (Roche Diagnostics, Mannheim, Germany), DNase (Wako Pure Chemical Industries, Osaka, Japan) and RNase (Sigma-Aldrich, St. Louis, MO), and Pepsin (Sigma-Aldrich) in that order. The enzyme-treated cell wall was finally washed with distilled water. WTA was then extracted from the cell wall fraction with 100 ml of 10% trichloroacetic acid (TCA) at 4 °C for 72 h and precipitated with ethanol (5 vol) at 4 °C for 48 h. The precipitate was purified by repetition of dissolution with TCA and precipitation with ethanol to remove low-molecular-weight substances. After it was washed with ethanol several times, until the precipitate became powdered, the WTA was dried in a centrifugal evaporator (CVE-2000, Tokyo Rikakikai, Tokyo). It was then fractionated on a DEAE-Toyopearl 650M (Tosoh, Tokyo) column in a gradient of water-0.5 M sodium chloride to remove neutral polysaccharides, and on a Sephadex G-75 (GE Healthcare, Buckinghamshire, UK) column (2.6 × 70 cm) in water to prepare homogeneous WTA. Since the WTA in this study contained glucose residues, the fractions containing WTA were detected by the phenol-H_2SO_4 method. The WTA-containing fractions were collected and lyophilized.

Preparation of monomeric units of WTA. Monomeric units of WTA were prepared as described by Archibald et al. The purified WTA was dissolved in 1 ml of 1 N sodium hydroxide and hydrolyzed at 100 °C for 3 h. After neutralization with 0.5 ml of 2 N hydrochloric acid, the hydrolysate was demineralized on a Sephadex G-25 (GE Healthcare) column. Sugar phosphates in the hydrolysate were analyzed by high-performance liquid chromatography (HPLC, LC-10 system, Shimadzu, Kyoto, Japan) on an Shodex Asahipac NH2P-50 4E (Showa Denko, Tokyo) column with 50 mM sodium-phosphate buffer (pH 4.4). The peaks of sugar phosphates were detected with the refractive index detector (RID-10A, Shimadzu), and were collected with a fraction collector (FRC-10A, Shimadzu). The sugar phosphates in the fraction were separated by charcoal chromatography on a charcoal powder-Celite 535 (1:1, w/w, Wako) column and then eluted with 50% ethanol. In this way WTA monomers were prepared from purified WTA.

Additionally, dephosphorylated monomers were prepared from the WTA monomers by enzyme treatment. The WTA monomers were dissolved in 1 ml of 50 mM glycine-sodium hydroxide buffer (pH 10.4) containing 0.5 U/ml alkaline phosphatase (Sigma) and incubated overnight at 37 °C. From the reaction mixture, dephosphorylated monomers were separated by charcoal chromatography under the same conditions, and the fraction absorbed to the column was collected and lyophilized. In this way the dephosphorylated WTA monomers were prepared.

Analytical methods. Constituent sugars of the dephosphorylated monomers were analyzed by HPLC on an Anasphere NH2P-50 4E column with water-acetonitrile (25:75, v/v). The monomers were hydrolyzed with 1 ml of 6 N hydrochloric acid at 100 °C for 3 h in a vacuum reaction tube. The hydrochloric acid in the hydrolysate was removed in a rotary vacuum evaporator (Tokyo Rikakikai). The dried residue was dissolved in a small amount of water and used in the analysis by HPLC. In this analysis, most of the ribitol in the monomers was changed to anhydroribitol by the acid hydrolysis, and the chromatogram of HPLC showed the peak of anhydroribitol instead of ribitol. Therefore, the ribitol content was determined by creating a calibration curve of the anhydroribitol. Optical rotation was analyzed on the digital polarimeter DIP-140 (Jasco, Tokyo) at 589 nm in distilled water. High-resolution mass spectra of the monomers were obtained by time-of-flight mass spectrometry (TOF-MS) using an AQUITY UPLC System coupled with an LCT Premier XE Mass spectrometer (Waters, Tokyo). Leucine (molecular weight, 131.1729) was used as the reference.

NMR spectra were recorded on an INOVA-500 (Varian, Palo Alto, CA) spectrometer in D_2O at ambient temperature with proton and carbon frequencies of 499.86 and 125.69 MHz respectively. Dioxane (δ: 6.75 ppm) and sodium 2,2-dimethyl-2-silapentane-5-sulfonate (δ: 0 ppm) were used as the references. H-H correlation spectroscopy (COSY) spectra were recorded with a spectral width of 1324 × 1324 Hz, eight repetitions, and 2 × 512 increments. H-C heteronuclear single quantum coherence (HSQC) spectra were recorded with a width of 6525 × 2142 Hz, a 2.0 s relaxation delay, eight repetitions, and 2 × 256 increments. Additionally, H-C constant time heteronuclear multiple-bond connectivity (CT-HMBC) spectra were recorded using pulse field gradient programs with a width of 6525 × 2142 Hz, a 2.0 s relaxation delay, 32 repetitions, and 256 increments. H-P HMBC NMR analysis was performed on an ALPHA-500 (Jeol, Tokyo) spectrometer with proton and phosphorus frequencies of 500.00 and 202.35 MHz respectively. The spectrum of F1 was recorded with a spectral width of 5070 × 1272 Hz, a 1.4 s pulse delay, and 32 times × 256 total scans.

Results

Preparation of WTA

The crude WTA extracted from the cell wall of L. plantarum NRIC 1068 was purified on the DEAE and Sephadex columns. A neutral polysaccharide with glucose and rhamnose has been reported in the cell wall of L. plantarum, but we did not detect it during the WTA purification process.

Preparation of monomeric units of WTA

HPLC profiles of an alkaline hydrolysate of WTA revealed two peaks of sugar phosphates (Fig. 1). These peaks were fractionated as fraction 1 (F1) and fraction 2 (F2) of the WTA monomers, and were purified by charcoal chromatography. After lyophilization, approximately 30 mg of each monomer was obtained from 200 mg of the purified WTA. Additionally, we used alkaline phosphatase to prepare dephosphorylated monomers from F1 and F2, and the digested monomers were isolated by charcoal chromatography. Approximately 10 mg of each dephosphorylated monomer (DPf1 or DPf2) was obtained from 20 mg of F1 or F2.

Structural analysis of DPf1 and DPf2

Component analysis of DPf1 and DPf2 by HPLC showed the presence of ribitol and glucose in both monomers. Additionally, there were no differences in the 1H and 13C NMR spectra of DPf1 and DPf2: these structures were entirely identical. Therefore, DPf1 and DPf2 were dealt with in the subsequent investigation as the same dephosphorylated monomer.

The content of ribitol and glucose in the hydrolysate of the dephosphorylated monomer was measured by HPLC analysis, and the molar ratio of ribitol to glucose was calculated to be approximately 1:2. Examination of the NMR spectra of the dephosphorylated monomer also showed two anomeric signals of glucose, at 5.18 and
5.08 ppm on $^1$H NMR and at 100.75 and 96.61 ppm on $^{13}$C NMR. These results suggest that the dephosphorylated monomer consisted of one molecule of ribitol and two glucose residues. The coupling constant of the anomeric signals was 3.90 Hz on $^1$H NMR and 170.37 Hz on $^{13}$C NMR. These values indicated that the glucose residues had $\alpha$-glycosidic linkages. Additionally, the high positive optical rotation of the dephosphorylated monomer ($\lbrack \alpha \rbrack_D^{20} +123.2$) also indicated the presence of $\alpha$-d-glycosidic linkages. Hence, the dephosphorylated monomer was assumed to be $\alpha$-d-diglucosyl ribitol.

On the basis of this structural assumption, three synthetic monomers, 2,3-, 2,4- and 3,4-$\alpha$-d-diglucosyl ribitol, were prepared to determine the steric configuration of the glucose residues of the dephosphorylated monomer (Nukada et al., in preparation). The structures of these synthetics were clear from the exclusive synthetic pathways, and we were able to distinguish them by NMR spectroscopy. On the basis of comparisons between the isolate and the chemical synthetics, the optical rotation and $^1$H and $^{13}$C NMR spectra of the isolate corresponded to that of 3,4-$\alpha$-d-diglucosyl ribitol. Thus the structure of the dephosphorylated monomer of WTA from the L. plantarum NRIC 1068 cell wall was identified as 3,4-$\alpha$-d-diglucosyl ribitol. The molecular formula of the dephosphorylated monomer was identified as C$_{17}$H$_{35}$O$_{15}$ by TOF-MS (m/z 557.1442 [M + H]$^+$, calculated for 557.1483).

The structures of F1 and F2 were analyzed on the basis of assignment of the dephosphorylated monomer, and the glucose residues were assigned on the basis of the two-dimensional NMR spectra of the monomers (Fig. 2). Although F1 and F2 were isolated as different peaks on the HPLC chromatogram, their dephosphorylation products were both determined to be 3,4-$\alpha$-d-diglucosyl ribitol. This indicated that the structural difference between F1 and F2 existed at the binding site of the phosphoric acid group. The coupling that occurred between carbon and phosphorus was useful in identifying the position of that group. Signals with coupling were extracted by observation of the $^{13}$C NMR spectra of F1 and F2 (Fig. 3B and C).

In the $^{13}$C NMR spectrum of F1, three signals with coupling appearing at 78.85 ($J_{CP} = 6.5$), 76.14 ($J_{CP} = 5.5$) and 61.77 (broad) ppm (Fig. 3B). These signals were assigned to C-3, C-2, and C-1 respectively, in the ribitol residue by two-dimensional NMR. Hence we suggest that the phosphoric acid of F1 was linked to C-2 of the ribitol residue. This suggestion was confirmed by examination of the H–P CT-HMBC spectrum, which revealed a correlation between phosphorus and H-2 in the ribitol residue (data not shown). Thus F1 was determined to be 3,4-$\alpha$-d-diglucosyl-1-phosphoryl ribitol (Fig. 4). The chemical shifts are listed in Table 2.

We then analyzed the structure of F2 by a similar procedure. In the $^{13}$C NMR spectrum of F2, only one signal with coupling appeared, at 66.57 (broad) ppm (Fig. 3C), and it was assigned to C-1 of the ribitol residue. Thus, F2 was determined to be 3,4-$\alpha$-d-diglucosyl-1-phosphoryl ribitol (Fig. 4). The chemical shifts are listed in Table 3. Although C-2 of the ribitol residue was invisible due to overlapping with the signal of C-4 of the glucose residues, the signal of C-2

<table>
<thead>
<tr>
<th>Table 1. $^1$H and $^{13}$C Chemical Shifts of the Dephosphorylated Monomer</th>
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<tbody>
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<td>Unit</td>
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<tr>
<td>------</td>
</tr>
<tr>
<td>Rbo</td>
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<tr>
<td></td>
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<tr>
<td>Gcl1 → 3</td>
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<td>Gcl1 → 4</td>
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probably showed coupling between carbon and phosphorus. The molecular formula of F2 was identified as C_{17}H_{33}O_{18}P by TOF-MS (m/z 557.1484 [M + H]^+, calculated for 557.1483).

**Discussion**

We used NMR spectroscopy to analyze two monomeric units (F1 and F2) prepared from ribitol-type WTA.
of *L. plantarum* NRIC 1068 cell walls. F1 and F2 were novel compounds, identified as 3,4-α-d-diglucosyl-2-phosphoryl ribitol and 3,4-α-d-diglucosyl-1-phosphoryl ribitol respectively, and the chemical shifts of these monomers were obtained as novel information on WTA monomers and discovered the presence of 3,4-α-d-diglucosyl ribitol as a monomeric structure. 15) The novel compounds, identified as 3,4-α/C11 of *L. plantarum* the structure of ribitol-type WTA in the cell walls of *L. plantarum* and discovered the presence of 3,4-α-d-diglucosyl ribitol as a monomeric structure. 15) The positions of the glucose residues were also confirmed by NMR spectroscopy in this study. In addition, we determined the positions of the phosphoric acid residues. The phosphoric acid residue of F1 was bound to C-2 of ribitol, and that of F2 was bound to C-1 of ribitol. Generally, the phosphoric acid residues of ribitol-type WTA bind to the terminal of ribitol, and the WTA consists of phosphodiester linkages between C-1 and C-5 of the ribitol. However, we obtained not only a 1-phosphoryl ribitol unit (F2) but also a 2-phosphoryl ribitol unit (F1). Hence we suggest that the WTA configuration of this strain consists of a complicated repeating unit. The existence of a WTA structure that contains two monomers differing in the position of the phosphoric acid residue is exceptional.

F1 and F2 were also characterized by a structure with two glucose residues. Ribitol-type WTA is present in the cell walls of *B. subtilis, S. aureus*, and *Listeria monocytogenes* as well as *L. plantarum*, and the WTA structures of these organisms have been elucidated, but multiple glucosylated monomers such as F1 and F2 are unknown outside *L. plantarum* NRIC 1068. So far, several rare WTA structures have been found in other organisms, which contain arabitol, erythritol, or mannitol in the main chain and are modified by α-acetylgalactosamine or 6-O-alanylglucose. These organisms have unique WTA structures with varied structural components. In contrast, *L. plantarum* appears to use only glucose and ribitol, the most general components of WTA, and to have a unique structure containing two glucose residues. The unique structure of the WTA in *L. plantarum* is created by modification with multiple glucose residues, which makes it possible, in theory, to expand the structural diversity of WTA in *L. plantarum* cell walls. This characteristic modification contributes to the diversity of WTA at the strain level.

The structural diversity provided by WTA probably correlates with functions of the cell wall, but the relationships between the structural diversity, especially the glycosyl residues, and the functions of WTA are not yet established. Neuhaus and Baddiley explained that many bacteriophages showed binding specificity for accessible WTA on cell surface of Gram-positive bacteria. Additionally, several studies of bacteriophages have reported a relationship between glycosyl residues in WTA and phage adsorption to the bacterial cell wall. Therefore, the WTA of *L. plantarum* are also clearly required for avoiding bacteriophage infection. The multiple glucosylated monomers of WTA of *L. plantarum* are suggested to be more useful against the infection by bacteriophages due to the structural diversity by modification with multiple glucose residues.

### Table 2. $^1$H and $^{13}$C Chemical Shifts of F1

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<th>1 (1a, 1b)</th>
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<th>3</th>
<th>4</th>
<th>5 (5a, 5b)</th>
<th>6 (6a, 6b)</th>
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<tr>
<td>Rbo (A)</td>
<td>$^1$H</td>
<td>3.95, 3.96</td>
<td>4.47</td>
<td>4.17</td>
<td>4.10</td>
<td>3.84, 3.86</td>
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<td>$^{13}$C</td>
<td>61.77 (broad)</td>
<td>76.14 (JCP = 5.5)</td>
<td>78.85 (JCP = 6.5)</td>
<td>79.98</td>
<td>62.32</td>
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<td>G1c(1 → 3) (B)</td>
<td>$^1$H</td>
<td>5.18</td>
<td>3.55</td>
<td>3.67</td>
<td>3.43</td>
<td>3.84</td>
<td>3.79, 3.88</td>
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<td></td>
<td>$^{13}$C</td>
<td>100.53</td>
<td>72.22</td>
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<tr>
<td>G1c(1 → 4) (C)</td>
<td>$^1$H</td>
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<td>73.64</td>
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### Table 3. $^1$H and $^{13}$C Chemical Shifts of F2

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<th>5 (5a, 5b)</th>
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<td>4.08, 4.12</td>
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<td>$^{13}$C</td>
<td>66.57 (broad)</td>
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<td>$^1$H</td>
<td>5.10</td>
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<td>3.41</td>
<td>3.84</td>
<td>3.76, 3.85</td>
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<td>$^{13}$C</td>
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<td>70.08</td>
<td>72.45</td>
<td>60.91</td>
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It is conceivable that the structural diversity seen in WTA is also present in other lactic acid bacteria, but little is known about the structures of these other species.\(^2\)\(^7\)\(^2\) Minimal knowledge of the WTA of *Lactobacillus buchneri* and *L. helveticus* has been obtained by chemical decomposition methods. Although the WTA of *L. plantarum* has been the most frequently studied among the WTAs of lactic acid bacteria, the monomeric structures of glycerol-type WTA have not yet been determined. Therefore, it is not too much to say that our knowledge of the WTA structure in lactic acid bacteria is still at a basic level. In this context, our elucidation of the structure of WTA in modern NMR techniques is a step toward understanding the importance of WTA in the cell wall of lactic acid bacteria.

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**References**