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Structures of Cell Wall Teichoic Acids of *Brevibacterium iodinum* VKM Ac-2106^T

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Abstract—Structures of two cell wall teichoic acids of *Brevibacterium iodinum* VKM Ac-2106^T were studied. The structure of mannitol teichoic acid described earlier was mainly confirmed. This polymer is 1,6-poly(mannitol phosphate) bearing β-D-glucopyranosyl residues at the C-2 of mannitol and pyruvic acid residues at the C-4 and C-5. The absolute configurations of D-mannitol and *S*-pyruvic acid were found. The following distinctions from the earlier described structure were found: unsubstituted 1,6-poly(mannitol phosphate) residues and residues substituted only by β-D-glucopyranosyl at the C-2 of mannitol but unsubstituted by pyruvic acid are present in the chain. The structure of glycerol teichoic acid present in the cell wall as a minor component (~7%) is also described. This acid is identified as 1,3-poly(glycerol phosphate) substituted at the C-2 of glycerol by 2-acetamido-2-deoxy-α-D-galactopyranosyl residues bearing *R*-pyruvic acid residues at the C-4 and C-6 of galactose. This polymer is for the first time described in the cell wall of Gram-positive bacteria.

Key words: *Brevibacterium*, teichoic acids, poly(glycerol phosphate), poly(mannitol phosphate), pyruvic acid acetal, NMR spectroscopy

Earlier the structures of cell wall polymers of *Brevibacterium iodinum* NCTC 9742 (= VKM Ac-2106^T) were studied by Anderton et al. [1]. It was shown that the major component is 1,6-poly(mannitol phosphate) substituted by β-glucopyranose at the C-2 and by pyruvic acid at the C-4 and C-5 of mannitol. The cell wall also contained a glycerol teichoic acid whose structure was not studied.

In the present work, we continued the studies of the carbohydrate fraction from the cell wall of *Brevibacterium iodinum* VKM Ac-2106^T in order to clarify the structure of glycerol teichoic acid, a minor component of the cell wall. The absolute configurations of mannitol and pyruvic acid in mannitol teichoic acid, the major component, also needed to be found.

MATERIALS AND METHODS

Brevibacterium iodinum VKM Ac-2106^T culture was sustained on corynebacterial agar (casein-peptone, 10 g; glucose, 5 g; yeast extract, 5 g; NaCl, 5 g; 2% agar; 1 liter H_2O, pH 7.0).

Biomass was grown aerobically on peptone-yeast medium in a shaker flask at 28°C to the mid-logarithmic phase [2]. Cells were harvested by centrifugation, washed with 0.95% NaCl, and used for isolation of cell walls. The latter were obtained from raw mycelium after its disintegration using a UZDN-1 supersonic disintegrator (Russia) as described in [3].

Chromatography and electrophoresis were performed on Filtrak FN-13 paper (Germany). To separate phosphoric esters, electrophoresis was performed in pyridine-acetate buffer (pH 5.5-5.6, 20 V/cm, 3-4 h). For descending paper chromatography, the following solvent systems were used: to separate monosaccharides and glycerol, *n*-butanol–pyridine–benzene–H_2O (5 : 3 : 1 : 3 v/v) (1); to separate monosaccharides, glycerol, and mannitol, *n*-butanol–acetic acid–H_2O (4 : 1 : 5 v/v) (2); to separate aminosaccharides, pyridine–ethyl acetate–acetic acid–H_2O (5 : 5 : 1 : 3) (3); for identification of pyruvic acid, amyl alcohol–5 M formic acid (1 : 1 v/v) (4) and *n*-propanol–2 M NH_4OH (7 : 3 v/v) (5).

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Teichoic acids and phosphoric esters were detected with Isherwood reagent; monosaccharides, with aniline phthalate; glycerol, mannitol, and monosaccharides, with 5% $AgNO_3$–ammonia solution; aminosaccharides, with ninhydrin.

Teichoic acids were extracted from cell wall with 10% trichloroacetic acid (1 : 10 w/v) at 4°C. After 24 h, the mixture was centrifuged, and the mycelium was extracted once more under the same conditions. The supernatants were pooled, dialyzed against distilled water, and lyophilized. The total preparation was thus obtained.

Acidic hydrolysis of cell wall and teichoic acid was performed under the following conditions: to study phosphoric esters, monosaccharides, and pyruvic acid, 2 M HCl, 3 h, 100°C; to study aminosaccharides, 6 M HCl, 3 h, 100°C.

To obtain glycosides, the preparation of teichoic acid was treated with 48% HF for 24 h at 4°C. HF was removed by lyophilization through a trap with NaOH. The products of hydrolysis were separated on a column (90 × 1.5 cm) with TSK 40S gel from Merck (Germany); elution was monitored using a differential refractometer from Knauer (Germany).

The ^{13}C-NMR spectra of the total preparation and monomers were recorded for solutions in 99.92% D_2O at 303 K. Chemical shifts were measured with respect to acetone as internal standard (δ 2.225 and 31.45 for ^{1}H and ^{13}C, respectively) and to 80% H_3PO_4 as external standard (δ 0.0 for ^{31}P). 2D spectra were recorded using a DRX-550 spectrometer from Bruker (Germany) applying the standard Bruker procedures. The spin lock time for TOCSY was 0.2 sec, and mixing time for ROESY was 0.1 sec. ^{1}H/^{13}C HMBC and ^{1}H/^{31}P HMQC were optimized for spin-coupling constants $J_{H,C}$ 8 Hz and $J_{H,P}$ 10 Hz.

RESULTS AND DISCUSSION

Glucose, mannitol, glycerol, and phosphoric esters of mannitol and glycerol were detected in acidic hydrolyzates of the cell wall of *B. iodinum* VKM Ac-2106^T.

Analysis of acidic hydrolyzates of the teichoic acids revealed mannitol mono- and diphosphates [4] and phosphate esters of glycerol as minor components, and also inorganic phosphate, glucose, mannitol, glycerol, galactosamine, and pyruvic acid.

Detailed chemical analysis of the products of alkaline and enzymatic degradation of the main component of mannitol teichoic acid was described earlier [1]. That is why we studied only certain aspects of the structure not discussed by the above mentioned authors.

The absolute configuration of D-mannitol was determined from the polarization rotation angle of the borate complex of mannitol isolated from the total preparation as described earlier [5].

Four fractions were obtained as a result of separation of the HF-hydrolyzate on a column. Based on the ^{13}C-NMR data, the first fraction contained the glycoside β-D-Glc*p*-(1→2)-Man-ol(4.5 Pyr) (monomer I), whereas the second fraction contained Man-ol(4.5 Pyr) (monomer II). These monomers were spectrophotometrically characterized (Tables 1 and 2). Two remaining fractions were identified as partially degraded polysaccharide and were not studied.

A preparation containing a mixture of teichoic acids was studied by NMR spectroscopy. ^{13}C- (Fig. 1) and ^{1}H-NMR spectra of the total preparation mainly coincided with the spectra of cell wall polymer of *B. iodinum* VKM Ac-2106^T, described and interpreted earlier [1] (Tables 1 and 2). The differences between the spectra were caused by the presence of a minor admixture in the preparation: 1,3-poly(glycerol phosphate) polymer with α-Gal*p*NAc-(4,6 Pyr) at the C-2 of Gro (characteristic signals C-1 at δ 96.7, C-2 at δ 50.8 and C-3′ Pyr at δ 26.4) and also by the presence in the main polymeric chain of unsubstituted 1,6-mannitol phosphate residues and the residues substituted only by β-Glc*p* at the C-2 of mannitol but not substituted by pyruvate.

To identify the positions of these residues, 2D COSY, TOCSY, ROESY, ^{1}H/^{13}C HSQC and HMBC, and also ^{1}H/^{31}P HMQC spectra were analyzed; in 1D ^{1}H and ^{13}C spectra this allowed interpretation of all the signals of four types of 1,6-mannitol phosphate residues, namely unsubstituted, substituted by β-Glc*p* at the C-2, substituted by pyruvate at the C-4,5, and substituted by β-Glc*p* at the C-2 and simultaneously by pyruvate at the C-4,5 (Tables 1 and 2). Based on the interpretation results, the substitution effects in ^{13}C-NMR spectrum were calculated, which can be further used to determine the relative absolute configuration of mannitol and the carbohydrate residue on substitution of the former at the C-2 or C-5 [6, 7].

The absolute configuration of *S*-pyruvic acid was determined as follows. In case of *S*-configuration of pyruvic acid at the C-4 and C-5 of D-mannitol (Fig. 2, *S*) its methyl group approaches the hydrogen atom at the C-6 of mannitol, which is sufficient for manifestation of nuclear Overhauser effect (NOE) in the PMR spectra. In case of *R*-configuration of pyruvic acid (Fig. 2, *R*), its methyl group approaches the hydrogen atom at the C-5 of mannitol. In 2D ROESY spectra of the total preparation and monomer I the CH_3/H-6,6′ correlation peaks were found (δ 1.70/4.19 and 1.69/3.88 for the total preparation and monomer I, respectively); this indicates *S*-configuration of pyruvic acid.

The structure of the minor (~7%) component, poly(glycerol phosphate), was established in the present work; the key signals in its ^{13}C-NMR spectrum are given above. The H-1- H-4 signals of the saccharide residue with the signal of anomeric proton at δ 5.18 were found in TOCSY spectrum. α-Configuration of the galactopyra-

Table 1. Chemical shifts in ^{1}H-NMR spectrum of mannitol teichoic acid of *Brevibacterium iodinum* VKM Ac-2106^T and monomers I and II

Residue	Chemical shift (δ), ppm							
	H-1	H-1'	H-2	H-3	H-4	H-5	H-6	H-6'
-1)-D-Man-ol-(6-*P*-	4.14	4.00	4.02	3.91	3.91	4.02	4.00	4.14
-1)-D-Man-ol-(6-*P*- 2) ↑	4.04	4.00	4.05	3.90	4.04	4.01	4.00	4.14
β-D-Gl*cp*-(1	4.64		3.32	3.51	3.41	3.42	3.91	3.73
-1)-D-Man-ol-(6-*P*- / \ 4 5 \ / *S*-Pyr	3.97	3.90	3.88	3.75 1.70	4.57	4.51	4.19	4.19
β-D-Gl*cp*-(1 ↓ 2)	4.64		3.32	3.51	3.41	3.42	3.91	3.73
-1)-D-Man-ol-(6-*P*- / \ 4 5 \ / *S*-Pyr	4.28	4.11	4.05	3.89 1.70	4.63	4.55	4.19	4.19
Monomer I								
β-D-Gl*cp*-(1 ↓ 2)	4.62		3.33	3.51	3.41	3.42	3.91	3.74
D-Man-ol / \ 4 5 \ / *S*-Pyr	3.96	3.80	3.91	4.03 1.70	4.43	4.26	3.83	3.83
Monomer II								
D-Man-ol / \ 4 5 \ / *S*-Pyr	3.82	3.63	3.70	3.64 1.69	4.58	4.42	3.88	3.88

nose residue was determined by analysis of the shape of correlation peaks. The position of the H-5 signal could not be determined from COSY and TOCSY spectra since the spin-spin coupling constant $J_{4,5}$ is very small. However, the alone standing H-3 signal of the residue (Table 3) produced a satisfactory 1D NOE spectrum with selective pre-irradiation of the H-3 signal. In the differential NOE spectrum the H-4 signal was observed at δ 4.09 and a triplet of approximately the same intensity in its vicinity (δ 4.07); this triplet was attributed to H-5 of the galactopyranose residue. In turn, correlation peaks for this signal with H-6 and H-6′ resonating at δ 3.99 and 4.04, respectively, were found in COSY spectrum. Analyzing correlation peaks in heteronuclear 2D HSQC spectra, positions of carbon atoms in the ^{13}C-NMR spectrum of this residue were found (Table 4). The position of the C-2 signal in the high-field area (δ 50.8) typical of the carbon atoms bound to the nitrogen atom indicated that the residue with galactopyranose configuration is an aminosaccharide. A minor signal at δ 23.5 typical of resonance of the acetamide methyl groups present in the ^{13}C-NMR spectrum of the total preparation indicated the presence of N-acetyl substituent at the C-2 of the residue, accounting that any signals in the resonance area of car-

Table 2. Chemical shifts in ^{13}C-NMR spectrum of mannitol teichoic acid of *Brevibacterium iodinum* VKM Ac-2106^{T} and monomers I and II

Residue	Chemical shift (δ), ppm**					
	C-1	C-2	C-3	C-4	C-5	C-6
-1)-D-Man-ol-(6-*P*-	68.1	70.8	69.7	69.7	70.8	68.1
-1)-D-Man-ol-(6-*P*- 2) ↑	66.7 (−1.4)	78.8 (+8.0)	71.0 (+1.3)	68.6 (−1.1)	70.8 (0)	68.1 (0)
β-D-Gl*cp*-(1	103.0	74.6	77.0	71.0	77.0	62.1
-1)-D-Man-ol-(6-*P*- / \ 4 5 \ / *S*-Pyr	67.5 (−0.6) 176.2	71.8 (+1.0) 106.5	69.0 (−0.7) 23.0	77.2 (+7.9)	78.8 (+8.0)	66.4 (−1.7)
β-D-Gl*cp*-(1 ↓ 2) -1)-D-Man-ol-(6-*P*- / \ 4 5 \ / *S*-Pyr	103.0 66.0 (−2.1) 176.2	74.6 79.2 (+8.4) 106.5	77.0 68.1 (−1.6) 23.0	71.0 77.6 (+7.5)	77.0 78.8 (+8.0)	62.1 66.4 (−1.7)
D-Man-ol*	64.4	72.1	70.5	70.5	72.1	64.4
Monomer I						
β-D-Gl*cp*-(1 ↓ 2) D-Man-ol / \ 4 5 \ / *S*-Pyr	102.7 61.2 (−2.8) 176.2	74.55 79.2 (+7.1) 106.8	77.0 68.4 (−2.1) 23.2	70.95 77.0 (+6.5)	77.1 80.8 (+8.7)	61.9 61.8 (−2.6)
Monomer II						
D-Man-ol / \ 4 5 \ / *S*-Pyr	64.1 (−0.3) 176.0	72.9 (+0.8) 106.3	69.7 (−0.8) 22.8	78.0 (+7.5)	80.5 (+8.4)	61.7 (−2.7)

* Chemical shifts for the standard D-mannitol sample.
** Substitution effects in D-mannitol residues are given in brackets.

bon atoms bound to the nitrogen atom except that at δ 50.8 are absent. A comparison of the chemical shifts of C-4 and C-6 of this residue with those of the corresponding nuclei in α-Gal*p*NAc [6] showed a low-field shift of the C-4 and C-6 signals (δ 1.8 and 3.7, respectively) and a high-field shift of the C-5 signal (δ 7.6). Shift direction and values coincided with the effects of 4,6-substitution of α-Gal*p* by *R*-pyruvic acid [7]. The presence of the latter in the total preparation was proved by a singlet at δ 1.57 in PMR spectrum, which corresponded with a minor signal at δ 26.4 in ^{13}C-NMR spectrum (correlation in HSQC spectrum). Comparable integral intensity of the signals of α-Gal*p*NAc and CH_3 of pyruvate in ^{13}C-NMR spectrum suggested localization of the latter on α-Gal*p*NAc residue. The value of chemical shift of the methyl group (δ 26.4) agrees with its equatorial orienta-

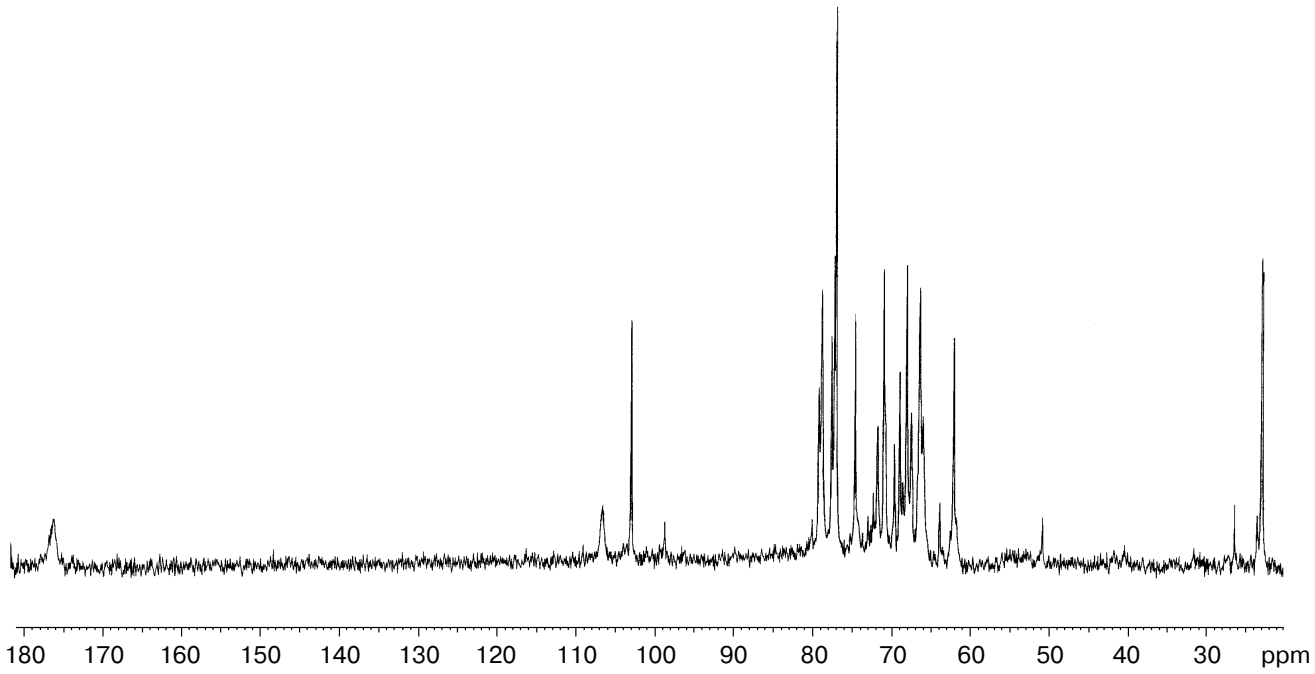


Fig. 1. 62.9 MHz ^{13}C-NMR spectrum of the total preparation of cell wall polymers of *Brevibacterium iodinum* VKM Ac-2106^T.

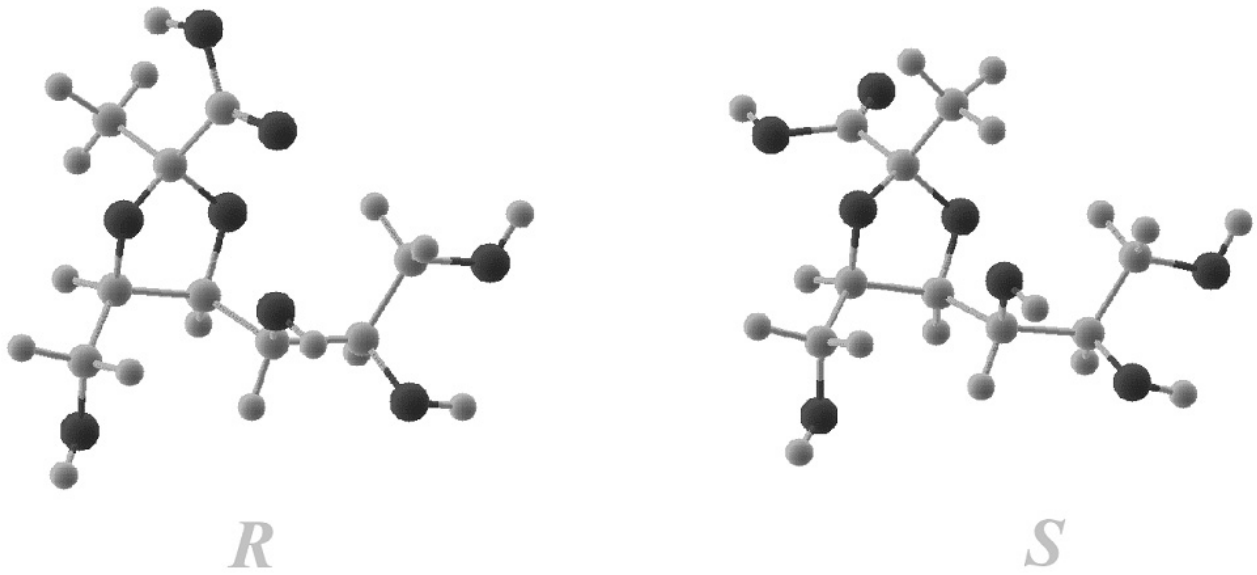


Fig. 2. Low-energy conformers of 4,5-*R*- and 4,5-*S*-pyruvates of D-mannitol.

tion in the six-membered cycle (*R*-configuration of pyruvate at the C-4,6 Gal*p*), whereas at the axial orientation (*S*-configuration) this signal would be shifted to the higher field (δ ~17) [7, 8]. The final evidence that the signals at δ 1.57 and 26.4 in ^{1}H- and ^{13}C-NMR spectra, respectively, belong to the pyruvic acid residue was obtained analyzing HMBC spectra: correlation peaks at δ 1.57/100.8 (H-3/C-2 of pyruvate [7]) and 1.57/175.3 (H-3/C-1 of pyruvate).

Analyzing the HSQC spectrum of the total preparation, minor correlation peaks were found, typical of H and C atoms of the residues of 1,3-poly(glycerol phosphate) chains and the same residues substituted by saccharide residue at the C-2 (Tables 3 and 4 and also [9]).

Table 3. Chemical shifts in ^{1}H-NMR spectrum of glycerol teichoic acid of *Brevibacterium iodinum* VKM Ac-2106^{T}

Residue	Chemical shift, ppm					
	H-1	H-2	H-3	H-4	H-5	H-6,6'
-1)-Gro-(3-*P*-	3.88; 3.95	3.0	3.88; 3.95			
-1)-Gro-(3-*P*- 2) ↑	4.01; 4.03	4.23	4.01; 4.08			
α-D-Gal*p*NAc-(1	5.18	4.30	4.24	4.09	4.07	3.99; 4.04
4 6 *R*-Pyr			1.57			

Table 4. Chemical shifts in the ^{13}C-NMR spectrum of glycerol teichoic acid of *Brevibacterium iodinum* VKM Ac-2106^{T}

Residue	Chemical shift, ppm					
	C-1	C-2	C-3	C-4	C-5	C-6
-1)-Gro-(3-*P*-	68.0	71.3	68.0			
-1)-Gro-(3-*P*- 2) ↑	66.3	77.0	66.8			
α-D-Gal*p*NAc-(1	98.7	50.8	67.3	71.7	64.1	66.1
4 6 *R*-Pyr	175.3*	100.8*	26.4			

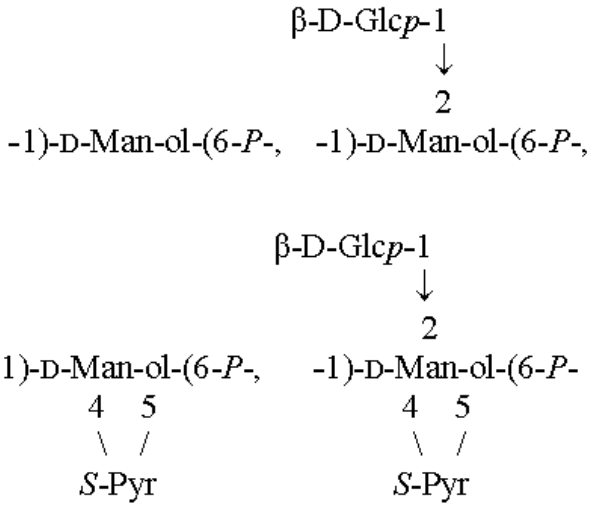
* From HMBC spectrum.

For the anomeric proton of α-Gal*p*NAc(4,6 Pyr), correlation peaks were found in the ROESY spectrum with H-2 of the same residue (δ 5.18/4.30), with the proton at C-2 (δ 77.0) of 2-substituted glycerol residue (δ 5.18/4.23) and with the protons at C-1 (δ 66.3) and/or C-3 (δ 66.8) of the same residue (δ 5.18/4.01-4.08).

The ROESY spectra confirm localization of α-Gal*p*NAc(4,6 Pyr) residues at the C-2 of glycerol residues in 1,3-poly(glycerol phosphate) chains.

So, the NMR study of the carbohydrate-containing fractions isolated from the cell wall of *B. iodinum* VKM Ac-2106^{T} indicated the presence of two teichoic acids varying in the nature of polyols.

The main polymer is 1,6-poly(mannitol phosphate). A part of monomeric units bear *S*-pyruvic acid at the C-4 and C-5 of mannitol, another part being substituted by *S*-pyruvic acid and also by β-glucopyranosyl residues at the C-2 of mannitol. Besides this, in teichoic acid we identified unsubstituted mannitol phosphate units and units substituted only by β-glucopyranose not detected earlier in this polymer. Thus, we conclude that mannitol teichoic acid of *B. iodinum* contains monomers of four types:



The structure of the second teichoic acid present in the cell wall as a minor (~7%) component was also established: it is 1,3-poly(glycerol phosphate) substituted at the C-2 of glycerol by 2-acetamido-2-deoxy-α-D-galactopyranose bearing *R*-pyruvic acid at the C-4 and C-6 of galactose. However, it should be mentioned that it remains unclear whether the minor polymer is heterogeneous, containing substituted as well as unsubstituted glycerol residues, or the total preparation contains minor homogeneous polymers.

Glycerol teichoic acid of such structure is for the first time described in the cell wall of Gram-positive bacteria.

Its should be mentioned that earlier *S*-pyruvic acid residues were identified in poly(glucosylglycerol phosphate) teichoic acid: on β-glucopyranose in *Nocardioides albus* VKM Ac-805^T [10] and on α-N-acetylglucosamine in *Brevibacterium linens* VKM Ac-2119 and Gk-3 [11], and *R*-pyruvic acid on α-galactopyranose in ribitol teichoic acid in *Nocardioides luteus* [12]. The presence in the same strain of two different teichoic acids bearing pyruvic acid residues was for the first time found in the cell wall. This fact is of special interest, accounting for the presence of these acetal substituents in teichoic acids of brevibacteria and also for the halotolerant properties of the organisms [13, 14].

The presence of poly(glycerol phosphate) chains along with mannitol phosphate or glycosylglycerol phosphate polymers in the cell walls and also heterogeneity of teichoic acid chains are typical of *Brevibacterium* genus; this holds for all the studied representatives of this genus [1, 4, 5, 11, 15, 16].

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