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Cell Wall Teichoic Acids of Two *Brevibacterium* Strains

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Abstract—Structurally identical teichoic acids were detected in cell walls of two soil isolates assigned to *Brevibacterium linens* based on phylogenetic data. Both cell walls contain unsubstituted 1,3-poly(glycerol phosphate) and poly(glycosylglycerol phosphate). Repeating units of the latter—α-D-Glc*p*NAc-(1→4)-β-D-Gal*p*-(1→1)-Gro—are bound by phosphodiester bonds including OH-3 of galactose and OH-3 of glycerol. Some of the N-acetylglucosamine residues have 4,6-pyruvic acid acetal, amounts of the latter in the two strains being unequal. Species-specificity of the structures of teichoic acids in the genus *Brevibacterium* is discussed.

Key words: *Brevibacterium*, teichoic acids, poly(glycosylglycerol phosphate), pyruvic acid acetal

Eleven species including a large heterogeneous group of organisms called *Brevibacterium linens* belong to the genus *Brevibacterium* [1-7]. Of all the species described, only *B. linens* strains are characterized by orange pigmentation of the colonies, so all orange-colored bacteria of the genus were assigned to this species.

While studying microorganisms isolated from long-standing frost sediments, rice paddies, and soil samples polluted with the waste of chemical and salt-mining industry, there was isolated a group of orange-colored strains phenotypically close to *B. linens*, and based on phylogenetic data three new species were described. Two isolates (Ac-2119 and GK-3) at the present stage of investigation are assigned to *Brevibacterium linens* [7].

Two *B. linens* strains, VKM Ac-2119 and GK-3, belonging to one genospecies with a type-strain *B. linens* VKM Ac-2112^T were close to each other in some physiological and biochemical features; however, they differ from the type-strain.

Earlier structural studies of cell wall teichoic acids of actinomycetes of other genus demonstrated that these polymers can be species-markers within a certain genus [8]; thus, the goal of this work was to study the structures of cell wall teichoic acids of the two *B. linens* strains, VKM Ac-2119 and GK-3; this can help to clarify their taxonomy in the future.

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MATERIALS AND METHODS

Strains were grown 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).

Biomasses of *Brevibacterium linens* VKM Ac-2119 and GK-3 were grown aerobically on peptone–yeast medium on a shaker at 28°C to the mid-logarithmic phase [9]. Cells were collected by centrifugation, washed with 0.95% NaCl, and used for isolation of cell walls. The latter were obtained from rough mycelium after its disintegration using a UZDN-1 supersonic disintegrator (Russia) as described in [10].

Chromatography and electrophoresis were performed on Filtrak FN-13 paper (Germany). To separate phosphoric esters and teichoic acids, 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, glycerol and glycosides, *n*-butanol–pyridine–benzene–H_2O (5 : 3 : 1 : 3 v/v) (1); to separate aminosaccharides, pyridine–ethyl acetate–acetic acid–H_2O (5 : 5 : 1 : 3 v/v) (2); for identification of pyruvic acid, amyl alcohol–5 M formic acid (1 : 1 v/v) (3) and *n*-propanol–2 M NH_4OH (7 : 3 v/v) (4).

Teichoic acids and phosphoric esters were detected with Ischerwood's reagent, monosaccharides with aniline phthalate, glycerol and monosaccharides with 5%

$AgNO_3$–ammonium solution, and aminosaccharides with ninhydrin.

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

Acidic hydrolysis of cell wall and teichoic acid was performed under the following conditions: to study phosphoric esters and monosaccharides, 2 M HCl, 3 h, 100°C; to study aminosaccharides, 6 M HCl, 3 h, 100°C; to obtain glycosides, 40% HF, 24 h, 4°C.

^{13}C-NMR spectra of 2-3% teichoic acid solutions were recorded at 30°C using a Brucker-DRX-500 spectrometer (Germany) with working C-frequency 75 MHz. Chemical shifts were measured with respect to acetone (δ 2.225 and 31.45 for ^{1}H and ^{13}C, respectively) as internal standard, and to 80% aqueous H_3PO_4 as external standard. 2D spectra were recorded using the standard Brucker (Germany) procedures.

RESULTS AND DISCUSSION

On acidic degradation, cell walls of *B. linens* VKM Ac-2119 and GK-3 formed the same compounds—galactose, glucose, and glycerol. Among phosphoric esters, glycerol mono- and diphosphates were identified; this can possibly indicate the presence of glycerol teichoic acids in the cell wall.

The polymers were extracted from cell walls of the two strains with 10% TCA, dialyzed, and lyophilized. The isolated preparations were qualitatively identical. Along with the same glycerol phosphoric esters, galactose, glucosamine, and pyruvate were identified in the products of their acidic degradation. To detect glycosylglycerol, the dephosphorylated repeating unit of teichoic acid, the polymer was treated with 40% HF for 24 h at 4°C. The glycoside fraction was collected by PC and pooled; two glycosides were obtained.

Chromatographic mobility of glycoside 1 was R_{Glc} = 0.8 in system 1. It gave typical staining with $AgNO_3$ and was not stained with aniline phthalate, and acidic degradation of glycoside 1 gave glycerol and galactose in approximately equimolar ratio. Periodate oxidation resulted in formation of 1 mol of formaldehyde per 1 mol of galactose; this indicated localization of the glycoside bond at the C-1 atom of glycerol in the presence of pyranose form of saccharide. The NMR data indicated that glycoside 1 is β-D-galactopyranosyl-(1→1)-glycerol (see below).

Chromatographic mobility of glycoside 2 was R_{Glc} = 0.37 in system 1. It also gave typical staining with $AgNO_3$ and was not stained with aniline phthalate. Acidic degradation yielded galactose, glucosamine, and glycerol. Data on chromatographic mobility and composition of glycoside 2 possibly indicated the presence of a disaccharide substituent. Glycoside 2 was obtained in small amounts and was not subjected to further investigations.

Chemical analysis data allow a dual interpretation. The presence of glycerol mono- and bisphosphate among the products of hydrolysis and also a small amount of glycoside 1 with localization of the glycoside bond at the C-1 of glycerol among the products of HF hydrolysis can indicate the presence of 2,3-poly(glycerol phosphate) chain negligibly substituted by glycosyl residues [11]. Another possible interpretation suggests the presence of various glycerol teichoic acids.

The preparation was studied by NMR. The ^{13}C-NMR spectra of preparations isolated from cell walls of *B. linens* VKM Ac-2119 and GK-3 were the same and only certain peak intensities differed (Fig. 1). According to the attached proton test (APT) [12], two signals with large peak intensity and integral intensities (δ 67.8 and 71.0) corresponded to the methylene and methyne groups, respectively. Based on the δ values and typical splitting of signals (doublet, 7 Hz, and triplet, 9 Hz, respectively), these two signals were assigned to the C-1,3 and C-2 of teichoic acid with chain 1,3-poly(glycerol phosphate) [13]. Along with these two signals, peaks with lower intensities were also observed, some of them being located in the characteristic spectral area. Thus, in the resonance area of carbon atoms bound with two oxygen atoms (δ 99.7-105.1), five signals from methyne groups with various intensities and one weak signal from ternary carbon atom (δ 102.1) were observed. In the resonance area of carbon atoms bound with a nitrogen atom, two signals (δ 55.0 and 55.4) were observed; in the high-field area, two signals from C-CH_3 groups (δ 23.6 and 26.1) were observed, and in the low-field area, there were two signals from carbon atoms of carbonyl groups (δ 175.3 and 176.5). In the ^{1}H-NMR spectrum in the low-field area (δ 4.4-5.1), six signals were observed; five of them had doublet splitting typical of anomeric protons of saccharides with α-gluco- or galactopyranose configuration (3.5 Hz, δ 4.97 and 5.00) and β-gluco- or galactopyranose configuration (8 Hz, δ 4.47, 4.49, 4.43). In the high-field spectral area, singlets from two acetyl groups (δ 2.105 and 2.115) and one CH_3-C group (δ 1.49) were observed. In the ^{31}P-NMR spectrum, two broad not fully resolved signals (δ 0.1 and 0.8) were observed.

Signals of the 1D ^{1}H-NMR spectrum were partly assigned using ^{1}H-^{1}H COSY, TOCSY, and ROESY (Fig. 2) and ^{1}H-^{31}P HMQC (Fig. 3) 2D spectra. Cross-peak analysis of these spectra showed that the carbohydrate part of the preparation includes two 2-acetamido-2-deoxy-α-glucopyranose (α-GlcNAc) residues and two β-galactopyranose (β-Gal) residues bearing phosphate groups at 3-position. The subspectra of two α-GlcNAc residues markedly differed from each other in chemical shifts of subsequent protons, whereas in subspectra of two β-Gal residues chemical shifts of four of seven protons

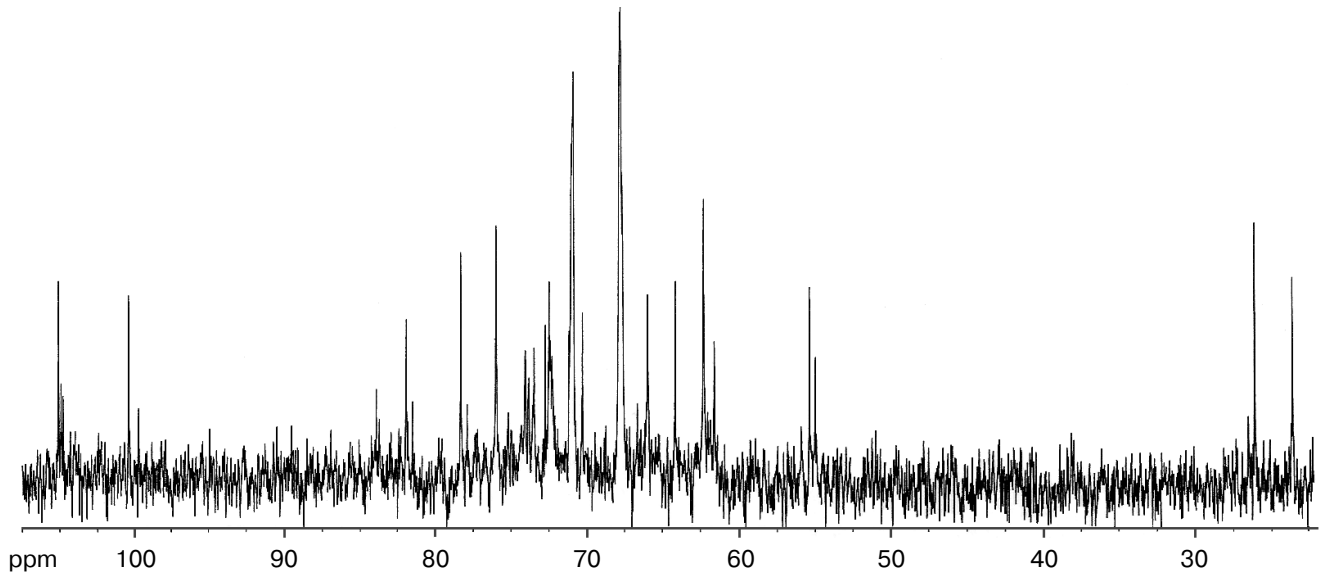


Fig. 1. ^{13}C-NMR spectrum of teichoic acid from *Brevibacterium linens* VKM Ac-2119 and GK-3.

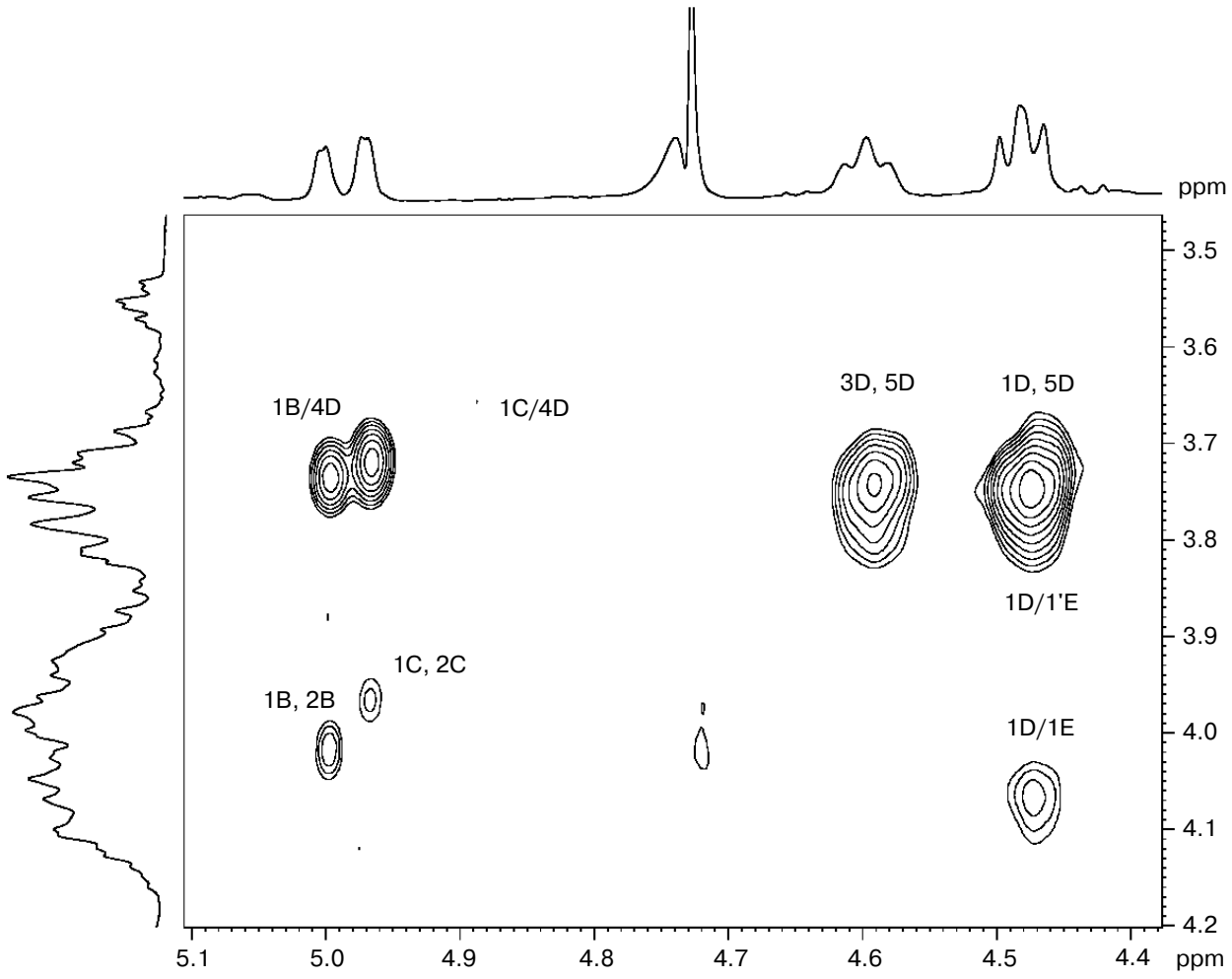


Fig. 2. Part of the ROESY spectrum of teichoic acid from *Brevibacterium linens* VKM Ac-2119 and GK-3.

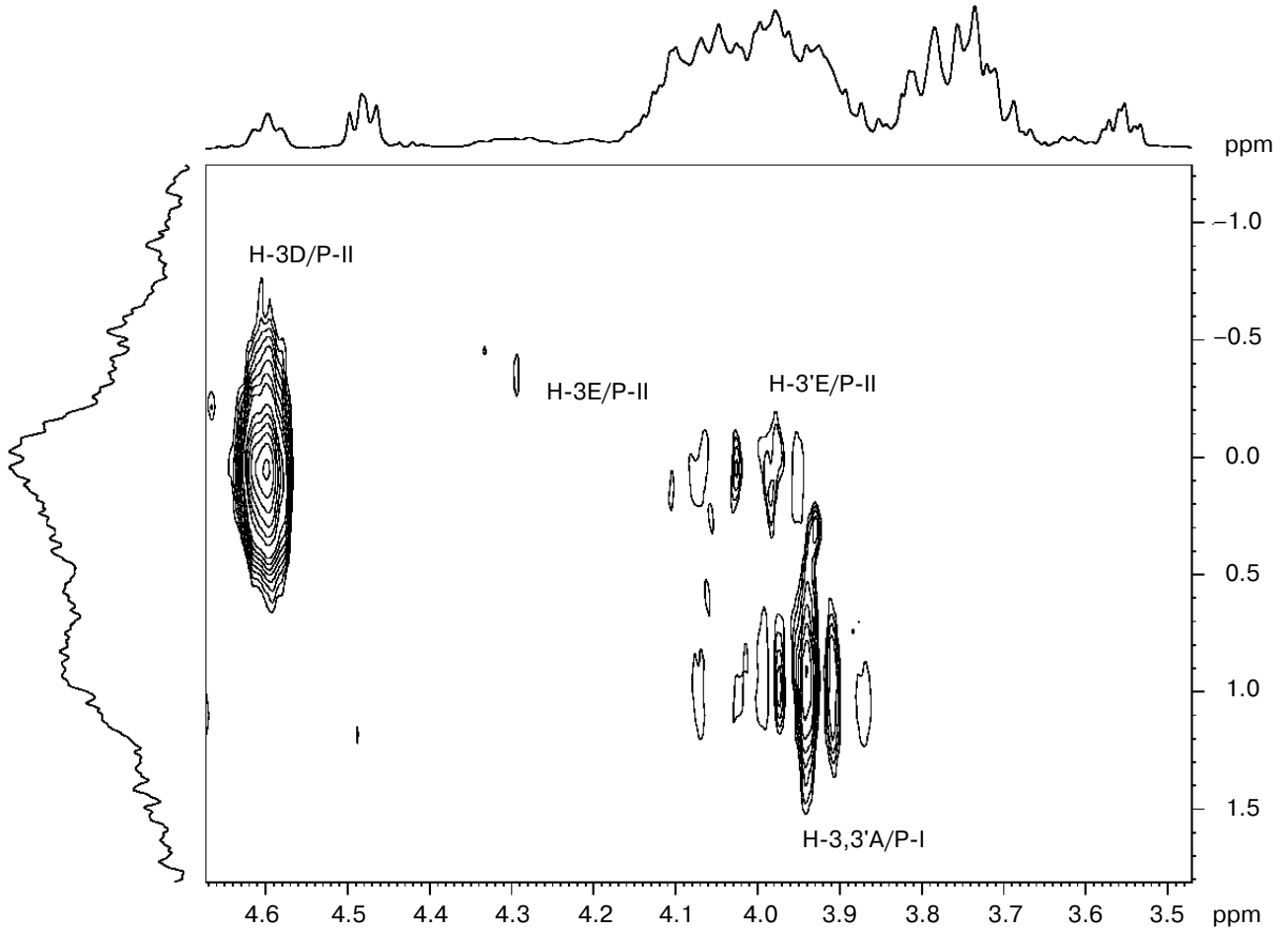
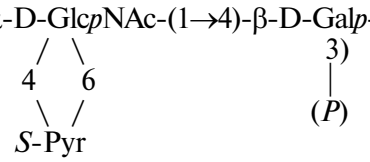


Fig. 3. Part of HMQC spectrum of teichoic acid from *Brevibacterium linens* VKM Ac-2119 and GK-3.

differed by not more than δ 0.03 and coincided for the remaining three protons (Table 1). Comparison of chemical shifts in ^{13}C-NMR spectrum obtained on analysis of the ^{1}H-^{13}C HSQC spectrum for two α-GlcNAc residues demonstrated that one of them is unsubstituted and another is 4,6-disubstituted (Table 2). Substitution effects in a disubstituted α-GlcNAc residue coincided completely with those for methyl-α-D-Glc*p*-4,6-(*S*)-Pyr [14]. ^{13}C-NMR subspectra of two β-Gal residues appeared to be almost identical: for C-1 and C-4, chemical shifts differed by no more than δ 0.2, whereas for the rest of the carbon atoms chemical shifts coincided. For C-4, the low-field chemical shifts indicated the presence of a substituent at this position. ^{1}H-^{13}C HSQC spectrum revealed ^{13}C signals of the glycerol residues bearing the phosphate group at the 3-position (δ 67.8 coincided with the C-1,3 signals of the abovementioned 1,3-poly(glycerol phosphate)) and the alkyl group at the 1st position, judging from δ 72.45 for C-1. Analysis of ^{1}H-^{13}C HSQC and ^{1}H-^{31}P HMQC spectra allowed assignment of the signals from the glycerol residues of the two abovementioned types in the ^{1}H-NMR spectrum of the preparation. Analysis of ROESY and ^{1}H-^{31}P HMQC spectra gave the bonding sequence of the residues. Anomeric protons of both saccharide residues with α-configuration of the glycoside center are spatially close to the H-4 of β-Gal residues (correlation peaks δ 5.00/3.73 and 4.97/3.71). The H-1 signals of β-Gal residues also correlate with the H-1 and H-1′ of glycerol residues in the ROESY spectrum (peaks 4.49; 4.47/4.06; 3.74). Based on these data and accounting for the position of phosphate groups obtained by analysis of the ^{1}H-^{31}P HMQC spectrum, the structure of the repeating unit of the second component of the studied preparation can be presented by the following formulae:

α-D-Glc*p*NAc-(1→4)-β-D-Gal*p*-(1→1)-sn-Gro-(3-*P*-
3)
|
(*P*)



Analyzing the 2D NMR spectra, the carbohydrate residue with minor signal from anomeric proton (δ 4.43) was identified as unsubstituted (terminal) β-D-galactopyranose residue. Its localization at the C-1 of glycerol at the end of the growing polymeric chain was identified by comparison of its subspectra in the spectra of polymer and glycoside 1 (Tables 1 and 2). Localization of the residue was directly proved by study of a lower-molecular-weight polymer preparation; correlation peaks (δ 4.33/3.71 and 4.33/72.9) were observed in its ROESY and HMBC spectra, respectively.

Thus, cell walls of *B. linens* VKM Ac-2119 and GK-3 contain two types of teichoic acids: unsubstituted 1,3-poly(glycerol phosphate) (type I) and poly(glycosylglycerol phosphate) (type II) [8]. Repeating units of the latter are bound to each other with phosphodiester bonds, which unite OH-3 of glycerol (E) and OH-3 of β-D-galactose (D). This structural base, (galactopyranosyl-(1→1)-glycerol phosphate), has side branches at the C-4 of galactose: 2-acetamido-2-deoxy-α-D-glucopyranosyl residues, both free (B) and bearing 4,6-pyruvic acid acetal at the C-4 and C-6 positions (C). 1,3-Poly(glycerol phosphate) is the main cell wall polymer.

It remains unclear whether these polymers are individual compounds or they are bound to each other, because electrophoresis in acetate-pyridine buffer (pH 5.5) showed that polymers move as one compound (m_{GroP} 0.98), whereas unsubstituted 1,3-poly(glycerol phosphate) has mobility m_{GroP} 1.2-1.3 under the same conditions [13].

Of the two suggestions, whether various glycosides comprise one chain or they are constituents of different polymers, the first seems to be more likely because a polymer bearing a pyruvic acid residue and thus possessing a larger negative charge should differ in electrophoretic mobility from a polymer without pyruvic acid acetal.

The presence of structurally different teichoic acids in the same cell wall is typical of brevibacteria [15-19].

Table 1. Chemical shifts in the ^{1}H-NMR spectrum of teichoic acid from *Brevibacterium linens* VKM Ac-2119 and GK-3 (δ, acetone signal at 2.225)

Residue		Hydrogen						
		H-1	H-1'	H-2	H-3	H-3'		
-1)-sn-Gro-(3-*P*-	(A,P-I)	4.01	3.94	4.09	4.01	3.94		
		H-1	H-2	H-3	H-4	H-5	H-6	H-6'
α-D-Glc*p*NAc-(1→	(B)	5.00	4.01[a]	3.875	3.555	4.05	3.82	3.82
α-D-Glc*p*NAc-(1→	(C)	4.97	4.12[a]	3.77	3.56	4.135	3.96	3.69
4,6-Pyr				1.49				
→4)-β-D-Gal*p*-(1→	(D)	4.49[b]	3.72[b]	4.60	3.73[b]	3.78[b]	3.80	3.78
		4.47[c]	3.73[c]		3.71[c]	3.75[c]		
3)-*P*	(P-II)							
→1)-sn-Gro-(3	(E)	H-1	H-1'	H-2	H-3	H-3'		
		4.06	3.74	4.09	4.05	3.97		
		H-1	H-2	H-3	H-4	H-5	H-6	H-6'
β-D-Gal*p*-(1→	(G)	4.43	3.58	3.68	3.93	3.70	3.78	3.82
		H-1	H-1'	H-2	H-3	H-3'		
→1)-sn-Gro-(3-*P*-	(E)	4.02	3.71	4.09	4.05	3.97		
		H-1	H-2	H-3	H-4	H-5	H-6	H-6'
β-D-Gal*p*-(1→	(G')	4.42	3.55	3.66	3.94	3.72	3.76	3.80
		H-1	H-1'	H-2	H-3	H-3'		
→1)-sn-Gro	(E')	4.02	3.70	3.95	3.68	3.63		

[a] CH_3CO (δ 2.105 and 2.115).
[b] For residues substituted by α-D-Glc*p*NAc.
[c] For residues substituted by α-D-Glc*p*NAc-4,6-Pyr.

Table 2. Chemical shifts in the ^{13}C-NMR spectrum of teichoic acid from *Brevibacterium linens* VKM Ac-2119 and GK-3 (δ, acetone at 31.45)

Residue		Carbon					
		C-1	C-2	C-3	C-4	C-5	C-6
-1)-sn-Gro-(3-*P*-	(A,P-I)	67.8	71.0	67.8			
α-D-Glc*p*NAc-(1→	(B)	99.7	55.0[a]	72.75	71.2	73.5	61.6
α-D-Glc*p*NAc-(1→	(C)	100.4	55.4[a]	70.3	78.3	64.0	66.1
4 6 / \ Pyr		176.5	102.1	26.1			
→4)-β-D-Gal*p*-(1→	(D)	105.1[b]	71.0	74.0	81.8[b]	76.0	62.3
3)		104.9[c]	70.9	67.8	81.6[c]		
| *P* |	(P-II)						
→1)-sn-Gro-(3	(E)	72.45	70.95	67.8			
β-D-Gal*p*-(1→	(G)	104.8	72.1	74.1	70.2	76.5	62.4
→1)-sn-Gro-(3-*P*-	(E)	72.9	71.0	67.8			
β-D-Gal*p*-(1→	(G')	104.9	72.2	74.2	70.1	76.6	62.3
→1)-sn-Gro	(E')	72.4	72.0	63.7			

[a] CH_3CO (δ 23.6 and 175.3).
[b] For residues substituted by α-D-Glc*p*NAc.
[c] For residues substituted by α-D-Glc*p*NAc-4,6-Pyr.

Three brevibacteria studied earlier were found to contain mannitol teichoic and glycerol teichoic acids simultaneously, whereas in cell walls of *B. linens* VKM Ac-2119 and GK-3 not less than two various glycerol teichoic acids are present simultaneously.

Teichoic acid from *B. linens* VKM Ac-2119 and GK-3 is the fourth structurally studied cell wall polymer of brevibacteria. Whereas the three earlier studied teichoic acids [17-19] are poly(polyol phosphate) polymers (type I), the teichoic acid presented in this work is a poly(glycosylpolyol phosphate) polymer (type II) [8]. The data can possibly be useful for clarification of species status of this bacterium, because in spite of the fact that according to the phylogenetic data, *B. linens* VKM Ac-2119 and GK-3 belong to the same group as a type-strain *B. linens* VKM Ac-2112, they have certain differences [7].

Comparative study of teichoic acids from two strains, Ac-2119 and GK-3, demonstrated that these polymers are structurally identical and differed only in the amounts of pyruvic acid. Teichoic acid from Ac-2119 contains more pyruvylated glycosaminyl residues (C) than teichoic acid of GK-3.

It should be noted that in three *Brevibacterium* species, pyruvic acid residues were found in teichoic acids, and 1,6-poly(mannitol phosphate) teichoic acid from *Brevibacterium antiquum* VKM Ac-2118^T has an additional phosphate group localized at OH-3 of each mannitol-phosphate residue of the main chain [18]. These data indicate that teichoic acids and, consequently, the cell surface of brevibacteria are more acidic compared with other Actinomycetales species, at least those studied to date. This fact is especially interesting if halotolerant properties of *Brevibacterium* species are considered [20]. Earlier it was found that the cell wall of an alkalophylic bacillus contained three polymers with clearly manifested anionic properties: polyglucuronic, teichuronic, and polyglutamine acids [21].

As for the taxonomy of teichoic acids from brevibacteria, the recent results indicate species-specificity of these polymers. Teichoic acids were the main cell wall polymers for four brevibacteria studied, whereas their structural peculiarities were unique for each particular species. Thus, teichoic acids from the brevibacteria studied are characteristic components of cell walls and can be used as taxonomic markers for these species.

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