ISSN 0026-2617, Microbiology, 2013, Vol. 82, No. 5, pp. 579–585. © Pleiades Publishing, Ltd., 2013.
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EXPERIMENTAL ARTICLES

Teichoic Acids of Three Type Strains of the *Bacillus subtilis* Group, *Bacillus mojavensis* VKM B-2650, *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* VKM B-2582, and *Bacillus sonorensis* VKM B-2652

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Received December 17, 2012

Abstract—Cell walls of three type strains of the *Bacillus subtilis* group, *Bacillus mojavensis* VKM B-2650, *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* VKM B-2582, and *Bacillus sonorensis* VKM B-2652, are characterized by the individual set of teichoic acids. All strains contained 1,3-poly(glycerol phosphates), unsubstituted, acylated with D-alanine, and glycosylated. The latter differ in the nature of the monosaccharide residue. Teichoic acids of *B. mojavensis* VKM B-2650^T and *B. amyloliquefaciens* subsp. *amyloliquefaciens* VKM B-2582^T contained α-glucopyranose, while those of *B. sonorensis* VKM B-2652^T contained β-glucopyranose and *N*-acetyl-α-D-glucosamine. Moreover, cell walls of *B. mojavensis* VKM B-2650^T contained a teichoic acid of poly(glycosylglycerol phosphate) nature with the following structure of the repeating unit: -4)-α-D-α-D-Glc*p*NAc-(1 → 3)]-Glc*p*-(1 → 2)-sn-Gro-(3-*P*-. The type strains have been characterized according to the composition of cell wall sugars and polyols. Application of teichoic acids (set and structure) as chemotaxonomic characteristics is discussed for six type strains of the *Bacillus subtilis* group. Polymer structures were determined by chemical and NMR spectroscopic techniques.

Keywords: Bacillus subtilis group, *Bacillus mojavensis* VKM B-2650^T, *Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* VKM B-2582^T, *Bacillus sonorensis* VKM B-2652^T, cell wall teichoic acids and sugars, NMR spectroscopy, taxonomy

DOI: 10.1134/S002626171305010X

Gram-positive endospore-forming bacteria, phylogenetically close to *Bacillus subtilis* (the *Bacillus subtilis* group) are the subject of many fundamental and applied studies [1, 2]. Taxonomic problems in differentiation between the species and subspecies of these bacteria at the phenotypic level have been noted repeatedly, which resulted in search for new chemical markers [1–4]. Teichoic acids and other cell wall glycopolymers may be such markers, as was shown for different genera of actinobacteria [5, 6].

Studies of the cell wall polymers of the *B. subtilis* group representatives demonstrated wide abudance of teichoic acids and their structural diversity [7–13]. Most of the studied strains were characterized by the simultaneous presence of several polymers with different structure [7–12]. In some cases, a phosphate-containing polymer, so called poly(glycosyl 1-phosphate), was present together with teichoic acids [7, 8].

Previous studies showed that teichoic acids (the set and the structure) allow clear differentiation of the type strains *B. subtilis* subsp. *subtilis* VKM B-501^T [8], *B. licheniformis* VKM B-511^T, *B. pumilus* VKM B-508^T [12], and *B. subtilis* subsp. *spizizenii* NRRL B-23049^T [13], belonging to the *B. subtilis* group. Studies of other strains of uncertain taxonomic affiliation, which have been assigned to *B. subtilis* on the basis of earlier taxonomic studies, resulted in a discovery of polymers structurally different from those of the type strains [9–11, 13].

This work is a continuation of the studies of the taxonomic significance of anionic cell wall polymers, and concerns the study of teichoic acid of the type strains of the species *B. mojavensis* [14], *B. amyloliquefaciens* subsp. *amyloliquefaciens* [15], and *B. sonorensis* [16].

MATERIALS AND METHODS

Strains of *B. mojavensis* VKM B-2650^T (=DSM 9205), *B. amyloliquefaciens* subsp. *amyloliquefaciens* VKM B-2582^T (=VKPM B-9866), and *B. sonorensis* VKM B-2652^T (=DSM 13779) were studied.

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The cultivation conditions were as previously described [8].

Cell walls were obtained from ultrasonically disintegrated cells (UP100H Ultrasonic Disintegrator, Hielscher, Germany) by differential centrifugation [6].

Teichoic acids were extracted from cell walls with 10% trichloroacetic acid [6]. The resultant preparations were marked as B-2650, B-2582, and B-2652 and studied according to the established procedure [8, 17].

Phosphorus determination and acid hydrolysis of the cell walls and of teichoic acids preparations were performed according to [17].

Electrophoresis and paper chromatography (with Filtrak FN 3), separation and detection of products of cell wall and teichoic acids degradation (2 M HCl for 3 h at 100°C) such as monosaccharides, glycerol, and its phosphate esters, were carried out as described elsewhere [6, 10].

The NMR spectra of the preparations were recorded with an Avance 600 spectrometer (Bruker, Germany) in 99.96% D_2O at 30°C. Sodium salt of 3-(trimethylsilyl)-3,3,2,2-tetradeuteropropionic acid (δ_H 0.0, δ_C 1.6) and 80% phosphoric acid (δ_P 0.0) were used as internal and external standards for chemical shifts calculation. Two-dimensional NMR analyses were performed using the standard techniques and software from the Bruker company. For the TOCSY and ROESY experiments the mixing time and the spin-lock time were 100 and 150 ms, respectively. The $^1H/^{13}C$ and $^1H/^{31}P$ 2D HMBC experiments were optimized for the coupling constant of 5 Hz.

RESULTS

Acid hydrolysis of the cell walls and teichoic acid preparations B-2650, B-2582, and B-2652 yielded identical products: glycerol mono- and bisphosphates, inorganic phosphate, glycerol, and glucose. In addition, glucosamine was detected in preparations B-2650 and B-2652.

Electrophoresis of B-2650, B-2582, and B-2652 preparations revealed the presence of fractions with different electrophoretic mobility, which suggested the presence of more than one polymer.

The complete structure, including the monomer composition, position of the phosphodiester and glycoside bonds, as well as configuration of the latter, were detected by NMR spectroscopy analysis of the one-dimensional (^1H, ^{13}C and ^{31}P), two-dimensional homonuclear ($^1H/^1H$ COSY, TOCSY, ROESY), and heteronuclear ($^1H/^{13}C$ HSQC, HMBC and $^1H/^{31}P$ HMBC) spectra.

The ^{13}C NMR spectra of the preparations differed significantly both in the carbon atoms signals and their relative intensity (Fig. 1, Table 1).

The analysis of the preparations B-2650, B-2582 and B-2652 spectra revealed the presence of 1,3-poly(glycerol phosphates) with unsubstituted (Polymer 1), acylated with alanine (Polymer 2), and glycosylated (Polymers 3 to 5) hydroxyl at C2 of the glycerol. The phosphate groups at O1 and O3 of glycerol residue in the polymers were identified by the presence of the H1,3/P correlation peaks in the $^1H/^{31}P$ HMBC spectra (Fig. 2). In some cases, the ^{13}C NMR spectra showed splitting of the C1,3 glycerol signals into a doublet and of the C2 signals into a triplet due to the constant values of the spin-spin interaction of ^{13}C and ^{31}P atoms through two or three simple communication, respectively.

The hydroxyl acylation with an alanine residue at C2 glycerol position (Polymer 2) was accompanied by a characteristic low-field chemical shift of the glycerol H2 proton and a relatively soft low-field effect for the C2 residue (Table 1). In all the preparations, except for B-2652, alanine was also detected as the terminal *O*-acyl group of the 1,3-poly(glycerol phosphate) chain.

The B-2582 and B-2650 preparations had α-glucopyranose as the glycosylation residue (Polymer 3) and B-2652 one had β-glucopyranose, partially (17%) *O*-acylated by the hydroxyl at C6 with alanine residues (Polymer 4).

The absolute configuration of glycerol residues was assigned on the basis of the existing conceptions of teichoic acid biosynthesis, according to which sn-glycerol-3-phosphate residues are incorporated into the poly(glycerol phosphate) chain [18]. Some of the works devoted to the study of glycosylation enzymes demonstrated incorporation of D-hexoses into teichoic acid molecules [19], which corroborates our data on determination of the absolute configuration of monosaccharides in analogous polymers [11].

The preparation B-2652 contained, in addition to the main components, a small amount (up to 5% of total polymer content) of 1,3-poly(glycerol phosphate) with 2-acetamide-2-deoxy-α-D-glucopyranose residues (α-D-Glc*p*NAc) at O2 of glycerol (Polymer 5, Table 1).

A distinctive feature of the B-2650 preparation was the presence of poly(glycosylglycerol phosphate) with the following structure of the repeating unit (Polymer 6):

$$\begin{array}{l} \text{-4)-α-D-Glc}p\text{-(1} \rightarrow \text{2)-sn-Gro-(3-}P\text{-} \\ \qquad\qquad\qquad\quad 3) \\ \qquad\qquad\qquad\quad \uparrow \\ \text{α-D-Glc}p\text{NAc-(1} \end{array}$$

Localization of phosphodiester bonds in the repeating unit resulted from the analysis of $^1H/^{31}P$ HMBC spectrum of the B-2650 preparation, which revealed the H4 α-Glc^{II}/P^{VI} and H3 Gro^{VI}/P^{VI} correlation peaks

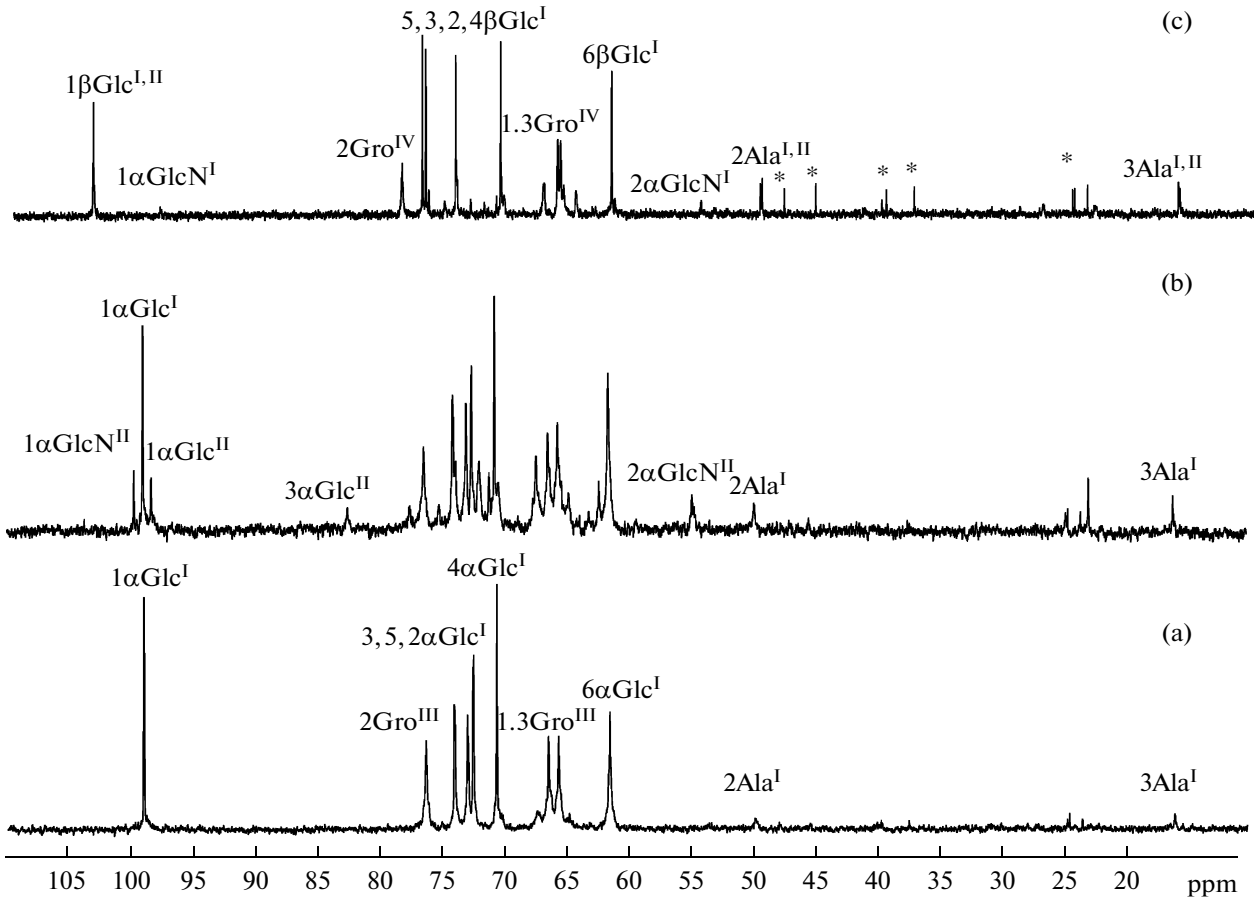

Fig. 1. The ^{13}C NMR spectra of *B. amyloliquefaciens* subsp. *amyloliquefaciens* VKM B-2582^T (a), *B. mojavensis* VKM B-2650^T (b), and *B. sonorensis* VKM B-2652^T (c) teichoic acids. Arabic numerals correspond to the carbon atoms numbers of the residues indicated according with the numbers in Table 1. Asterisks correspond to low molecular weight impurities.

(Fig. 2). The sequence of the residues in the chain was determined by the presence of peaks at C1 α-Glc^{II}/H2 Gro^{VI} and at C1 α-GlcN^{II}/H3 α-Glc^{II} in the $^1H/^{13}C$ HMBC spectrum (Fig. 3). The aforementioned structure of the teichoic acid is described for the first time.

DISCUSSION

This paper reports the composition of the cell wall sugars, polyols, and teichoic acids of *B. mojavensis, B. amyloliquefaciens* subsp. *amyloliquefaciens* and *B. sonorensis* type strains, belonging to the *B. subtilis* group for the first time.

Glucose and glycerol may be possible chemotaxonomic markers for the strains VKM B-2650^T, VKM B-2652^T, and VKM B-2582^T, glucosamine may be a marker for VKM B-2650^T and VKM B-2652^T.

Comparing the results obtained in this work with the previously published data on the type strains *B. subtilis* subsp. *subtilis, B. pumilus*, and *B. licheniformis*, belonging to the *B. subtilis* group [8, 12], shows that all strains are characterized by an individual set of phosphate-containing polymers (Table 2) and differ from each other on the basis of the "composition and structure of the cell wall anionic glycopolymers."

Teichoic acids of the poly(glycerol phosphate) nature, unsubstituted or substituted with D-alanine at O2 position of the glycerol residues, are characteristic for all the strains studied.

Glycosylated 1,3-poly(glycerol phosphates) were discovered in five out of six strains studied (Table 2). The polymers of *B. mojavensis* VKM B-2650^T, *B. amyloliquefaciens* subsp. *amyloliquefaciens* VKM B-2582^T, and *B. subtilis* subsp. *subtilis* VKM B-501^T, which are close by the 16S rRNA and the "housekeeping genes" sequences [4, 20, 21], contain α-glucopyranose. The cell wall of *B. sonorensis* VKM B-2652^T, which is phylogenetically distant from the strains mentioned above [4], contained 1,3-poly(glycerol phosphate) with β-glucopyranose and a small amount of the polymer substituted with *N*-acetyl-α-glucosamine (similar to the main polymer of *B. pumilus* VKM B-508^T [12]). The strains *B. sonorensis* VKM B-2652^T and *B. licheniformis* VKM B-511^T, which are most closely related

Table 1. Chemical shifts in ^{13}C NMR spectra (δ_C TSP −1.6) and ^{1}H (δ_H TSP 0.0, italics) of *B. mojavensis* VKM B-2650^T, *B. amyloliquefaciens* subsp. *amyloliquefaciens* VKM B-2582^T and *B. sonorensis* VKM B-2652^T cell wall teichoic acids

Residue		C1 *H1, 1'*	C2 *H2*	C3 *H3, 3'*	C4 *H4*	C5 *H5*	C6 *H6, 6'*
Polymer 1 (*B. mojavensis* VKM B-2650^T, *B. amyloliquefaciens* subsp. *amyloliquefaciens* VKM B-2582^T, *B. sonorensis* VKM B-2652^T)							
-1)-sn-Gro-(3-*P*-	**Gro^I**	67.4 *3.98, 3.92*	70.5 *4.05*	67.4[a] *3.98, 3.92*			
Polymer 2 (*B. mojavensis* VKM B-2650^T, *B. amyloliquefaciens* subsp. *amyloliquefaciens* VKM B-2582^T, *B. sonorensis* VKM B-2651^T)							
-1)-sn-Gro-(3-*P*-	**Gro^{II}**	64.8	75.3	64.8[b]			
2)		*4.12, 4.10*	*5.36*	*4.12, 4.10*			
|							
Ala-(1	**Ala^I**	171.1	50.1 *4.29*	16.5 *1.63*			
Terminal fragment of Polymer 2 (*B. mojavensis* VKM B-2650^T, *B. amyloliquefaciens* subsp. *amyloliquefaciens* VKM B-2582^T)							
sn-Gro-(3-*P*-	**Gro^{II}**	66.4	70.9	67.7			
1)		*4.64, 4.44*	*4.18*	*4.00, 3.88*			
|							
Ala-(1	**Ala^t**	171.6	50.0 4.27	16.5 *1.61*			
Polymer 3 (*B. mojavensis* VKM B-2650^T, *B. amyloliquefaciens* subsp. *amyloliquefaciens* VKM B-2582^T)							
-1)-sn-Gro-(3-*P*-	**Gro^{III}**	66.5	76.5	65.7[c]			
2)		*4.04, 4.06*	*4.12*	*4.06,*			
↑				*4.02*			
α-D-Glc*p*-(1	**αGlc^I**	99.0 *5.17*	72.6 *3.54*	74.1 *3.76*	70.8 *3.41*	73.1 *3.91*	61.8 *3.88, 3.77*
Polymer 4 (*B. sonorensis* VKM B-2652^T)							
-1)-sn-Gro-(3-*P*-	**Gro^{IV}**	66.0	78.9	66.3[d]			
2)		*4.09, 3.98*	*4.19*	*4.05, 4.05*			
↑							
β-D-Glc*p*-(1	**βGlc^I**	103.5 *4.65*	74.6 *3.33*	77.0 *3.53*	71.0 *3.40*	77.2 *3.46*	62.1 *3.91, 3.73*
↑							
β-D-Glc*p*-(1	**βGlc^{II}**	103.4	74.6	76.8	71.0	74.4	66.0
6)		*4.67*	*3.33*	*3.55*	*3.46*	*3.72*	*4.66, 4.45*
|							
Ala-(1	**Ala^{II}**	171.6	50.1 *4.28*	16.7 *1.62*			
Polymer 5 (*B. sonorensis* VKM B-2652^T)							
-1)-sn-Gro-(3-*P*-		66.7	77.3	66.0[e]			
2)	**Gro^V**	*4.03, 4.00*	*4.06*	*4.04, 4.00*			
↑							
α-D-Glc*p*NAc-(1	**αGlcN^I**	98.3 *5.10*	55.1[f] *3.94*	72.4 *3.81*	71.5 *3.48*	73.5 *3.94*	62.0 *3.89, 3.80*

Table 1. (Contd.)

Residue	C1 *H1, 1'*	C2 *H2*	C3 *H3, 3'*	C4 *H4*	C5 *H5*	C6 *H6, 6'*
Polymer 6 (*B. mojavensis* VKM B-2650^T)						
sn-Gro-(3-*P*-	62.4	77.6	65.4[g]			
2) **Gro^{VI}**	*3.76, 3.74*	*3.96*	*4.06, 4.01*			
↑						
-4)-α-D-Glc*p*-(1 **αGlc^{II}**	98.3	72.1	82.6	74.0[d]	72.0	61.6
3)	*5.16*	*3.73*	*3.91*	*4.16*	*3.95*	*3.91, 3.82*
↑						
α-D-Glc*p*NAc-(1 **αGlcN^{II}**	99.7	55.0[h]	72.0	71.2	73.9	61.8
	5.09	*3.99*	*3.84*	*3.48*	*4.01*	*3.88, 3.78*

Phosphorus signal at δ_P
[a] +0.8;
[b] +0.5;
[c] +0.5;
[d] +0.7;
[e] +0.2;
[f] CH_3CON at δ_C 23.6 and 176.0 and δ_H 2.08;
[g] +0.1;
[h] CH_3CON at δ_C 23.4 and 175.8 and δ_H 2.08.

phylogenetically [4], significantly differed, however, in teichoic acid composition and structure (Table 2).

The differences between the strains were also related to the presence/absence of a disaccharide 1-phosphate polymer or a teichoic acid of poly(glycosylglycerol phosphate) nature in the cell wall (Table 2).

The α- and β-glycosylated 1,3-poly(glycerol phosphates) and poly(glycosylglycerol phosphates) were previously found in several other *B. subtilis* group strains, the taxonomic position of which remains unclear [7, 9–11].

The O2 glycerol ester-bonded D-alanine residues were reported as characteristic elements for the struc-

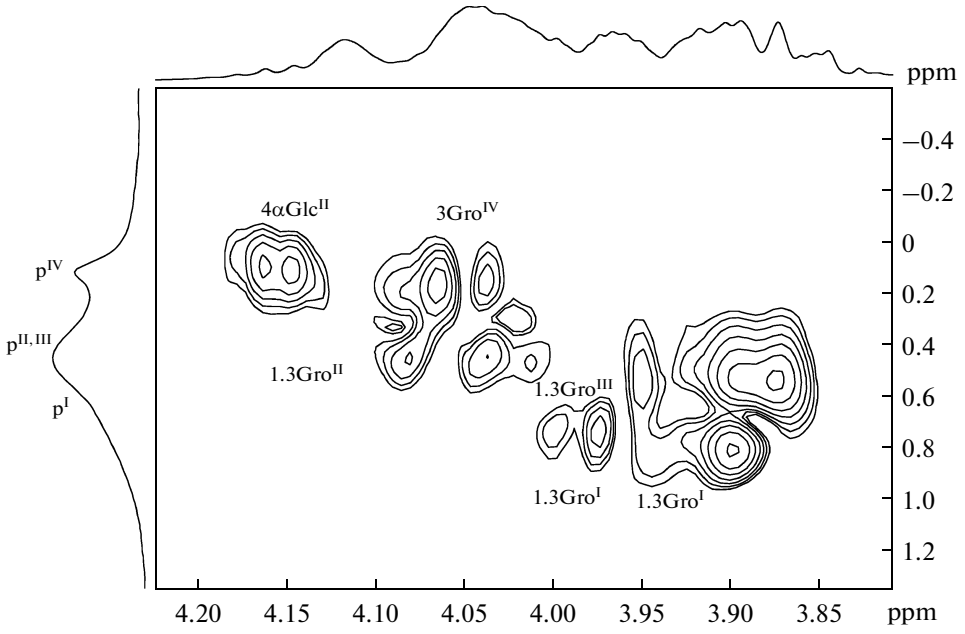

Fig. 2. Two-dimensional heteronuclear $^1H/^{31}P$ HMBC spectrum of *B. mojavensis* VKM B-2650^T teichoic acids. Arabic numerals correspond to proton numbers of the residues indicated according with the numbers in Table 1.

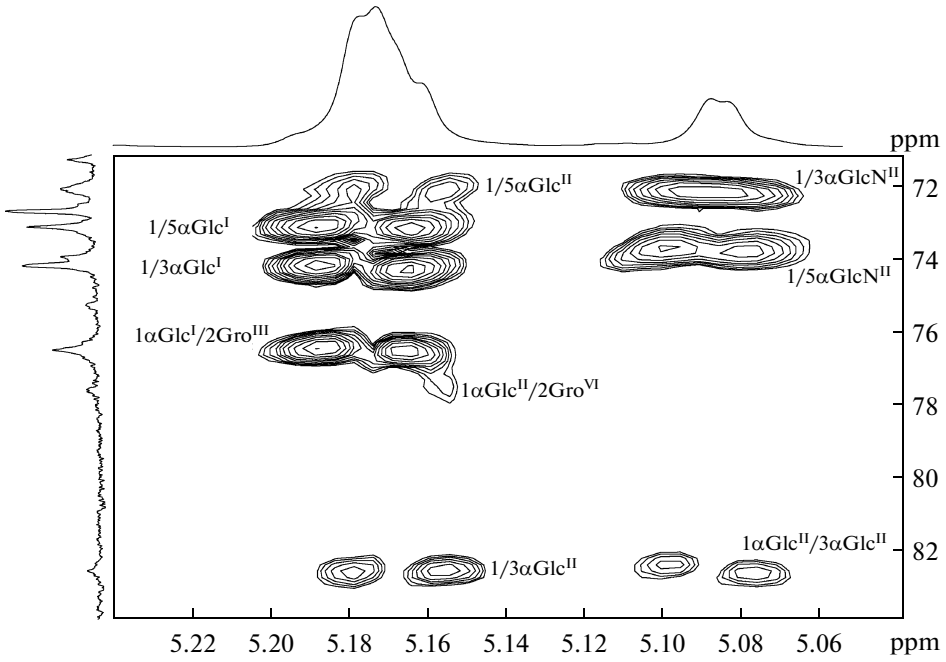

Fig. 3. Two-dimensional heteronuclear $^{1}H/^{13}C$ HMBC spectrum of *B. mojavensis* VKM B-2650^{T} teichoic acids. Arabic numerals before the slash correspond to the numbers of protons and after the slash to the numbers of carbon atoms in the residues indicated according with the numbers in Table 1.

ture of bacillar teichoic acids [7, 12, 18]. The D-alanine was also detected at O6 of glucose residues in the *B. sonorensis* VKM B-2652^{T} polymer. It was not found earlier in teichoic acids of bacilli. However, alanine in this position was previously reported in teichoic and lipoteichoic acids of *Staphylococcus epidermidis* RP62A and D group streptococci [22, 23], as well as in the *O*-polysaccharides of gram-negative bacteria [http://www.glyco.ac.ru/bcsdb3/].

Thus, the study of the cell wall polymers of the type strains of six species of the *B. subtilis* group demonstrated that the combination and structure of teichoic acids together with other chemotaxonomic characteristics, including fatty acid composition [3, 4, 14], the nature of the teichoic acids polyol [13], and the presence of the specific lipopeptide [4], may serve for phenotypic differentiation of the species. Investigation of teichoic acids of organisms and

Table 2. Cell wall phosphate-containing polymers of the B. *subtilis* group type strains

Phosphate-containing polymers teichoic acid/substituent and disaccharide 1-phosphate polymer	*B. subtilis* subsp. *subtilis* VKM B-501 [8]	*B. mojavensis* VKM B-2650	*B. amyloliquefaciens* subsp. *amyloliquefaciens* VKM B-2582	*B. licheniformis* VKM B-511 [12]	*B. sonorensis* VKM B-2652	*B. pumilus* VKM B-508 [12]
1,3-Poly(glycerol phosphate)	◆	◆	◆	◆	◆	◆
1,3-Poly(glycerol phosphate)/D-Ala	◆	◆	◆	◆	◆	◆
1,3-Poly(glycerol phosphate)/α-D-Glc*p*	◆	◆	◆			
1,3-Poly(glycerol phosphate)/α-D-Glc*p*NAc					◆	◆
1,3-Poly(glycerol phosphate)/β-D-Glc*p*					◆*	
Poly(glycosylglycerol phosphate)**		◆				
Poly(galactosylglycerol phosphate)***				◆		
Disaccharide 1-phosphate polymer****	◆					

* D-Ala at O6 position of certain β-Glc*p* residues.

** Structure of the poly(glycosylglycerol phosphate) repeating unit: -4)-α-D-[α-D-Glc*p*NAc-(1 → 3)]-Glc*p*-(1 → 2)-sn-Gro-(3-*P*-. The structure is described for the first time.

*** Structure of the repeating unit of poly(galactosylglycerol phosphate): -6)-α-D-[Gal*p*-(1 → 2)-sn-Gro-(3-*P*- [12].

**** Structure of the repeating unit of disaccharide 1-phosphate polymer: -6)-β-D-Glc*p*-(1 → 3)-α-D-Gal*p*NAc-(1-*P*- [8].

groups of organisms not previously investigated in this respect, also reveals immense structural diversity of these natural compounds.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research (grant no. 10-04-01747) and the Federal Program "Research and Development of the Priority Courses of Scientific and Technological Complex of Russia for 2007–2013" (State Contract no. 14.518.11.7069).

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Translated by M. Sokolov