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EXPERIMENTAL ARTICLES

Carbohydrate-Containing Cell Wall Polymers of Some Strains of the *Bacillus subtilis* Group

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Abstract—A comparative study of the structures of carbohydrate-containing cell wall polymers isolated from the strains of the *Bacillus subtilis* group was performed by means of chemical and NMR spectroscopic methods. Polymers of different structure were revealed, namely, 1,3-poly(glycerol phosphates) with β-glucopyranose in *Bacillus subtilis* strains VKM B-520, VKM B-723, and VKM B-763 (= VKM B-911); 1,5-poly(ribitol phosphate) with α-glucopyranose in *B. subtilis* strains VKM B-722 and VKM B-922 (the structure is reported for the first time); and simultaneously two polymers in *B. subtilis* VKM B-761, 1,5-poly(ribitol phosphate) with β-glucopyranose and the disaccharide 1-phosphate polymer with the following repeating unit: –6)-α-D-Gal*p*-(1-*P*-4)-β-D-Glc*p*NAc-(1–, in which the hydroxyls at C3 and C6 of glucosamine residues are partially O-acetylated (the structure is reported for the first time). Heterogeneity of the *B. subtilis* group is confirmed by variations in the structure and composition of the cell wall polymers. The cell surface polymers are useful for discrimination of closely related bacilli strains and are cell wall marker components that may be an indispensable element of the *Bacillus subtilis* group taxonomy along with the genomosystematic methods.

Keywords: Bacillus subtilis, cell wall, teichoic acid, disaccharide 1-phosphate polymer, taxonomy, NMR spectroscopy.

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The *Bacillus subtilis* group (complex) comprises species and subspecies of extremely high homology in terms of their phenotype and phylogenetic characteristics. Bacilli of the *B. subtilis* group are mostly nonpathogenic and therefore are widely used as producers of proteases, surface-active agents, in genetics studies and other areas of science and biotechnology [1]. The problem of differentiating between closely related organisms and isolation of new species and subspecies therefore is gaining importance. The work in this direction has been carried out for some time. From nine to ten species referred to the *B. subtillis* group were described in the past 20 years [1–3]. For example, in 1989 a number of *B. subtilis* var. *niger* strains producing a black pigment were assigned to the species *B. atrophaeus* [4], and in 1999 two subspecies *B. subtilis* ssp. *subtilis* and *B. subtilis* ssp. *spizizenii* were described on the basis of DNA–DNA homology and qualitative composition of cell wall polymers [5]. Comparative analysis of 16S rRNA genes is inefficient for the *B. subtilis* group strains, since the level of nucleotide sequence homology between strains of different species reaches 100% [2, 3]. To discover new taxa within the *B. subtilis* group, a more reliable method of multilocus phylogenetic analysis of the genes encoding protein synthesis has been proposed [3, 6]. Based on multilocus gene analysis, as well as the presence of a previously unidentified lipopeptide and the cell fatty acid composition, a new subspecies *B. subtilis* ssp. *inaquosorum* was described [3]. Although the modern approach to prokaryote taxonomy is genome-oriented, phenotypic characteristics remain indispensable for analysis. Chemical characteristics of the cells, such as the polymers of the cell wall and cytoplasmic membrane, gain particular importance. Only a combination of phenotypic and phylogenetic methods may provide an approach to natural classification of microorganisms [6].

The previously described structures of the cell wall polymers of some representatives of *B. subtilis* were studied mainly by chemical methods [7–10]. The structure of teichoic acid of *B. subtilis* var. *niger* WM [11] identified using NMR spectroscopy is an exception. Investigation of carbohydrate-containing poly-

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Table 1. List of studied *B. subtilis* strains (VKM)

B. subtilis strains	Collection of the strain origin	Author's strain name	Strain presence in other collections
B-520	INMIA	"*B. mesentericus niger*"	Absent in other collections
B-722	NCIB	"*B. subtilis* var. *aterrimus*"	NCIB 8055, ATCC 6461, CN 2192
B-723*	NCIB	"*B. subtilis* var. *niger*"	NCIB 8056, ATCC 6537
B-761	CCM	"*B. subtilis* var. *aterrimus*"	CCM 116, IFO 3214, NBRC 3214
B-763*	CCM	"*B. subtilis* var. *niger*"	CCM 878, CIP 52.79
B-911*	CIP	"*B. subtilis* var. *niger*"	CIP 52.79, CCM 878
B-922	INMIA	"*B. subtilis* var. *aterrimus*"	CIP 51.54

* The modern name of the strain, according to the StrainInfo bioportal (http://www.straininfo.net), is *B. atrophaeus*.

mers is presently impossible without the application of nondestructive research methods, including NMR spectroscopy.

The goal of the present work was to study the structure and composition of anionic carbohydrate-containing polymers as marker components of the cell wall to distinguish between closely related strains of the *B. subtilis* group at the phenotypic level.

MATERIALS AND METHODS

Seven *B. subtilis* strains from the All-Russian Collection of Microorganisms (VKM) were used in the present work: *B. subtilis* VKM B-722, VMK B-922, VKM B-761, VKM B-520, VKM B-723, VKM B-763, and VKM B-911 (Table 1). Strains *B. subtilis* VKM B-763 and VKM B-911 are subcultures of the same strain. The cultures were grown under aerobic conditions at 28°C to the midexponential growth phase, as has been previously described [12].

Amplification and sequencing of 16S rRNA gene fragments and phylogenetic analysis of the nucleotide sequences of 16S rRNA gene fragments were performed as described [13].

Cell wall isolation, quantification of alanine hydroxamate, phosphorus forms, degradation and dephosphorylation of polymers were performed as previously described [13–15].

Teichoic acids were isolated from the cell walls by fractional extraction with 10% trichloroacetic acid (two to three times for 24 h at 4°C) [13–15], and the sugar 1-phosphate polymer was extracted with a citrate buffer [12]. Then the supernatants were pooled, dialyzed against water, and lyophilized to obtain the polymer preparations.

Electrophoresis and paper chromatography. High-voltage electrophoresis was performed in pyridine–acetate buffer (**A**) (pH 5.5–5.6, 20 V/cm, 3–4 h). Descending paper chromatography (PC) to separate the products of acidic cell walls and preparations degradation was performed in the following solvent systems: *n*-butanol–pyridine–benzene–H_2O (5 : 3 : 1 : 3, vol/vol) (**B**), pyridine–ethyl acetate–acetic acid–H_2O (5 : 5 : 1 : 3, vol/vol) (**C**), and *n*-buthanol–AcOH–H_2O (4 : 1 : 5, vol/vol) (**D**) [13, 14]. Teichoic acids and phosphate esters were detected with Ischerwood's reagent; monosaccharides, with aniline phthalate; polyols and monosaccharides, with 5% $AgNO_3$ in aq ammonia; and amino acids, with ninhydrin.

NMR spectra of the preparate solutions in D_2O were recorded on a DRX-500 Bruker spectrometer (Germany) at 30°C. TSP (δ_H 0.0) and acetone (δ_C 31.45) were used as internal chemical shift standards, and 80% phosphoric acid was used as an external standard (δ_P 0.0). Two-dimensional spectra were recorded and processed using the standard techniques recommended by the manufacturer. The spin-lock times in TOCSY and HMQC-TOCSY experiments were 200 and 150 ms, respectively. The mixing time in ROESY experiments was 200 ms. HMBC and $^1H/^{31}P$ HMQC experiments were optimized for constant values of $J_{H,C}$ 8 and $J_{H,P}$ 10 Hz, respectively.

RESULTS

Nucleotide sequences of 16S rRNA gene fragments of strains VKM B-722 and B-922 (about 500 bp) and VKM B-520, B-723, B-763, and B-911 (1467 bp) were determined.

Comparative analysis of the data obtained for the strains under study and those for the type species revealed the highest homology (99–100%) between strains VKM B-520, B-723, B-763, and B-911 and the species *B. atrophaeus* and *B. vallismortis*; between strain VKM B-922 and *B. atrophaeus*; and between strains VKM B-722 and B-761 and *B. subtilis* ssp. *subtilis*.

Cell wall polymers. Cell walls and the preparations of carbohydrate-containing polymers were studied according to a conventional scheme [14]. In acidic cell wall hydrolysates of strains VKM B-722, B-761, and B-922 and in isolated preparations [14], the products characteristic of ribitol teichoic acid were detected, while in those from strains VKM B-520, B-723, B-763, and B-911, products characteristic of glycerol teichoic acid with 1,3-type of phosphodiester

Table 2. Characteristics of cell wall preparations and phosphorus-containing polymers of *B. subtilis* strains (VKM) under study

Strains	P^{*}_{TA}	Products of acid hydrolysis of cell walls and teichoic acids (2 M HCl, 3 h, 100°C)		m^{**}_{GroP}
		Phosphoesters	Monosaccharides	
B-723	1.1	GroP, $GroP_2$	Glc	0.8
B-763 (=B-911)	1.1	idem	idem	idem
B-720	1.0	idem	idem	idem
B-722	2.8	Rib-olP, Rib-olP_2, AnRib-olP	Glc	0.7
B-922	1.9	idem	idem	idem
B-761	1.9	Rib-olP, Rib-olP_2, AnRib-olP	Glc, Gal, GlcN	0.68 1.24

Notes: GroP and $GroP_2$, glycerol monophosphate and diphosphate; Rib-olP and Rib-olP_2, ribitol monophosphate and diphosphate; AnRib-olP, anhydroribitol phosphate.

* Teichoic acid phosphorus in % to cell wall weight;

** Electrophoretic mobility of phosphorus-containing polymers in comparison to glycerol phosphate.

bond were found [16] (Table 2). Glucose was present in the cell walls of all the bacilli under study, and VKM B-761 contained glucose, galactose, and glucosamine (Table 2).

In contrast to all other strains, electrophoresis of the B-761 preparation (buffer **A**) produced two fractions differing by mobility and monosaccharide composition (systems **B** and **C**), suggesting the presence of two polymers (Table 2). The presence of glucose identified in B-520, B-722, B-723, B-763, B-911, and B-922 preparations (system **B**) may indicate glycosylation of the polyol phosphate units in teichoic acids.

Hydroxyl aminolysis of teichoic acid preparations revealed trace amounts of O-ester bound alanine in all strains.

Complete polymer structure, including the configuration of the glycoside centers and position of phosphodiester bonds, was determined by NMR analysis. Teichoic acids with ribitol- and glycerol phosphate residues in the integral chain were identified in the preparations. A disaccharide 1-phosphate polymer was also detected. NMR study results are presented in Tables 3 and 4 and Figs. 1–3. NMR analysis of the preparations B-520, B-723, B-763, and B-911 con-

Table 3. Chemical shifts of ^{13}C, ^{1}H, and ^{31}P in NMR spectra of teichoic acid isolated from cell walls of *B. subtilis* strains VKM B-722 and VKM B-922

Residue	Chemical shifts (δ_C acetone 31.45, δ_H TSP 0.0)					
	C-1 *H-1,1'*	C-2 *H-2*	C-3 *H-3*	C-4 *H-4*	C-5 *H-5,5'*	C-6 *H-6,6'*
Polymer						
-1)-Rib-ol-(5-*P*-	68.5	71.4	71.1	79.2	66.2	
4)	*4.09**	*3.95*	*3.97*	*4.11*	*4.15**	
↑	*4.01*				*4.11*	
α-D-Glc*p*-(1	99.0	72.9	74.4	71.0	73.3	61.9
	5.10	*3.57*	*3.77*	*3.42*	*3.97*	*3.85, 3.77*
*Fragment **A***						
-*P*-1)-Rib-ol	68.3	71.7	71.2	81.2	62.1	
4)	*4.08***	*3.91*	*3.92*	*3.91*	*3.83*	
↑	*4.00*				*3.78*	
α-D-Glc*p*-(1	99.2	72.9	74.3	71.0	73.5	61.9
	5.05	*3.54*	*3.72*	*3.39*	*3.87*	*3.80, 3.73*
*Glycoside **B***						
Rib-ol						
4)	*4.11*	*3.95*	*3.98*	*3.95*	*4.11*	
↑	*3.99*				*3.99*	
α-D-Glc*p*-(1						

Notes: * ^{31}P at $\delta_P + 1.1$.

** ^{31}P at $\delta_P + 1.0$.

Table 4. Chemical shifts of ^{1}H and ^{13}C in the NMR spectra of *B. subtilis* VKM B-761 cell wall disaccharide 1-phosphate polymer

Residue	Chemical shifts (δ_C aceton 31.45, δ_H TSP 0.0)					
	C-1 *H-1*	C-2 *H-2*	C-3 *H-3*	C-4 *H-4*	C-5 *H-5*	C-6 *H-6,6'*
Polymer						
⟶6)-α-D-Gal*p*-(1-*P*- (***C***)	97.4[1,2], 97.2[3]	69.7	70.6	70.2	71.7[4], 71.3[5]	68.6
	5.59[1], *5.56*[2], *5.46*[3]	*3.82*	*3.82*	*3.97*	*4.17*[4],*4.11*[5]	*3.93*, *3.81*
						61.9
-4)-β-D-Glc*p*NAc-(1⟶ (***D***)	102.3	56.8[6]	75.1	75.5	76.5	
	4.70	*3.76*[6]	*3.78*	*4.01*	*3.57*	*3.93*, *3.83*
-4)-β-D-Glc*p*NAc-6OAc-(1⟶ (***D'***)	102.3	56.6[6]	74.6	75.9	74.2	64.8[7]
	4.73	*3.78*[6]	*3.76*	*4.10*	*3.90*	*4.54*, *4.39*
-4)-β-D-Glc*p*NAc-3,6OAc-(1⟶ (***D''***)	101.7	55.3[6]	75.9[7]	73.2	73.9	64.6[7]
	4.89	*3.90*[6]	*5.17*[7]	*4.37*	*3.81*	*4.52*, *4.40*[7]
Disaccharides						
⟶6)-α-D-Gal*p*-(1-OH (***C***$_{\alpha}$)	93.8	69.8	70.5	70.6	70.5	70.3
	5.25	*3.79*	*3.84*	*3.95*	*4.16*	*3.99*, *3.78*
⟶6)-β-D-Gal*p*-(1-OH (***C***$_{\beta}$)	97.2	73.3	74.1	70.1	75.2	70.5
	4.56	*3.48*	*3.63*	*3.89*	*3.79*	*3.96*, *3.78*
β-D-Glc*p*NAc-(1⟶ (***D***)	103.1	56.9[8]	75.2	71.4	77.3	62.2
	4.58	*3.72*[8]	*3.56*	*3.45*	*3.47*	*3.94*, *3.76*
β-D-Glc*p*NAc-6OAc-(1⟶ (***D'***)	103.1	56.9[8]	75.0	71.2	75.2	64.8[9]
	4.60	*3.73*[8]	*3.56*	*3.54*	*3.67*	*4.45*, *4.33*[9]

Notes: [1, 2, 3] Values for ***C*** residue bound to [1]***D***, [2]***D'***, or [3]***D''*** residue.

[4, 5] Values for the ***C*** residue glycosylated with [4]***D*** and ***D'*** or [5]***D''*** residue.

[6] $\underline{C}H_3\underline{\underline{C}}ON$ at $\delta_{\underline{C}}$ 23.8, 23.5 and $\delta_{\underline{\underline{C}}}$ 176.1, 176.8; $C\underline{H}_3CON$ at δ_H *2.06*, *2.05*, *2.01*.

[7] $\underline{C}H_3\underline{\underline{C}}OO$ at $\delta_{\underline{C}}$ 22.0, 21.8 and $\delta_{\underline{\underline{C}}}$ 175.4, 174.8; $C\underline{H}_3COO$ at δ_H *2.16*, *2.15*, *2.12*.

[8] $\underline{C}H_3\underline{\underline{C}}ON$ at $\delta_{\underline{C}}$ 23.6 and $\delta_{\underline{\underline{C}}}$ 176.2; $C\underline{H}_3CON$ at δ_H *2.06*.

[9] $\underline{C}H_3\underline{\underline{C}}OO$ at $\delta_{\underline{C}}$ 21.7 and $\delta_{\underline{\underline{C}}}$ 175.6; $C\underline{H}_3COO$ at δ_H *2.16*.

firmed the presence of 1,3-poly(glycerol phosphate) substituted with β-glucopyranose at C2 hydroxyl of glycerol. Polymers of similar structure are common in cell walls of gram-positive bacteria, and the spectral data of a similar teichoic acid were published previously [16].

In the region of resonance of anomeric carbon atoms in ^{13}C NMR spectra of B-722 and B-922 preparations (Table 3, Fig. 1), an intense signal was observed at δ_C 99.0 and a minor one at δ_C 99.2. A number of signals of various intensity were present, some of them broadened (at δ_C 68.5 and 66.2) or split into doublets (at δ_C 99.2, *J* 9.8Hz, and at δ_C 71.4, *J* 7.5Hz).

In ^{1}H NMR spectrum, an intense proton signal was observed in the region of anomeric carbon atoms at δ_H 5.10 (doublet, $J_{H,H}$ 3.5 Hz). The signals of the rest of the protons were observed in the resonance region δ_H 3.4–4.2. The ^{31}P spectrum contained a wide signal centered at δ_P +1.1. One-dimensional spectra were deciphered using the two-dimensional techniques $^{1}H,^{1}H$ COSY, $^{1}H,^{13}C$ gHSQC, and $^{1}H,^{31}P$ HMQC. Analysis of the two-dimensional spectra revealed that the polymer repeating unit contains 1,5-poly(ribitol phosphate) residues with hydroxyl groups at C4 position substituted by α-D-glucopyranose (Table 3). Minor signals were related to the terminal units where C5 of ribitol was lacking a phosphate residue. The intensity of the minor signals increased with the preparation storage in deuterated water solution. Autodepolymerization was complete within one month at room temperature and pD 3 yielding a solution of teichoic acid ***A*** fragment with a phosphate residue localized at C1 hydroxyl of ribitol. The structure was established by analysis of the two-dimensional spectra mentioned above. Fragment ***A*** was dephosphorylated forming glycoside ***B***, the structure of which was confirmed by ^{1}H and ^{13}C NMR spectra. Taking into account the small contribution of the ribitol residue to

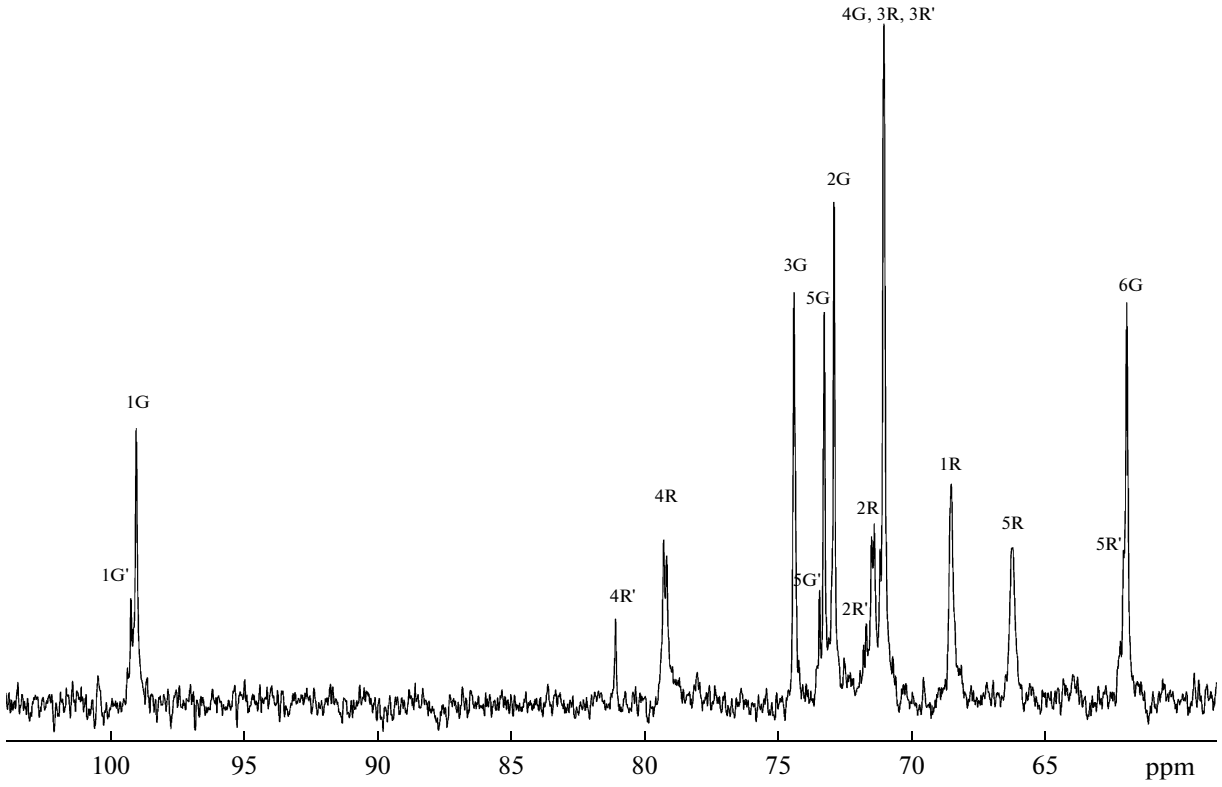

Fig. 1. The ^{13}C NMR spectrum of teichoic acid of *B. subtilis* VKM B-722 and VKM B-922 cell walls. The numerals indicate carbon atom positions in α-glucopyranose (G) and ribitol (R). Prime marks indicate the atoms of terminal units.

the glycoside optical rotation, the positive value of optical rotation of the glycoside [α_D^{18} +72.15, C = 1.58] proves the glucose D-configuration.

Thus, 1,5-poly(ribitol phosphate) teichoic acid substituted with α-D-glucopyranose at C4 hydroxyl of ribitol residue was detected in the preparations of B-722 and B-922. For the first time, α-glucose residue was registered in ribitol teichoic acids.

In B-761 preparation, two polymers were revealed, namely, teichoic acid 1,5-poly(ribitol phosphate) with the C4 hydroxyl substituted by β-D-glucopyranose and a disaccharide 1-phosphate polymer. ^{13}C NMR spectra and chemical shifts of a teichoic acid of similar structure were published previously [17]. Disaccharide 1-phosphate NMR analysis data are presented in Table 4. The ^{13}C NMR spectrum (Fig. 2) in the resonance region of the anomeric carbon atoms of sugars contained signals of unequal intensity at δ_C 101.7–102.3 and 97.2–97.4. In the resonance region of carbon atoms bound to a single oxygen, the APT spectrum [18] revealed the signals of substituted oxymethyl groups at δ_C 68.6, 64.8, and 64.6 and an unsubstituted oxymethyl group at δ_C 61.9. In the resonance region of carbon atoms bound to nitrogen, three signals at δ_C 56.8, 56.6, and 55.3 were observed. In the strongest field, the signals of methyl carbon atoms in N-acetyl and O-acetyl groups were present at δ_C 23.5–23.8 and 21.8–22.0, respectively. Signals of the carbonyl atoms of carbon of N- and O-acetates were localized in the region of δ_C 174.8–176.2. In the ^{31}P spectrum, three intense signals were observed at δ_P –2.5, –2.1, and –1.6 (Fig. 3).

In the ^1H NMR spectrum, a number of signals of varying intensity were observed in the low-field of resonance region of δ_H 4.7–5.6. Only some of them belonged to the protons of anomeric carbon atoms (see below). The signals of CH_3 groups in N- and O-acetates were observed in the form of singlets in the characteristic regions of δ_H 2.01–2.06 and 2.12–2.16, respectively. The ^1H NMR spectrum of the preparation was deciphered using the two-dimensional $^1H,^1H$ COSY, TOSCY, and ROESY experiments. Analysis of the two-dimensional spectra of B-761 preparation revealed that the polymer chain contained α-galactopyranose residues (α-D-Gal$_p$, residue ***C***, Table 4) and 2-acetamido-2-deoxy-β-*D*-glucopyranose (β-D-Glc*p*NAc, residue ***D***) partially acetylated at C6 hydroxyl (***D'***) or C6 and C3 hydroxyls (***D''***). Acetate position was determined by characteristic low-field shifts of protons by the carbon atom bearing an O-acetyl group (Table 4). Acetylation degree was

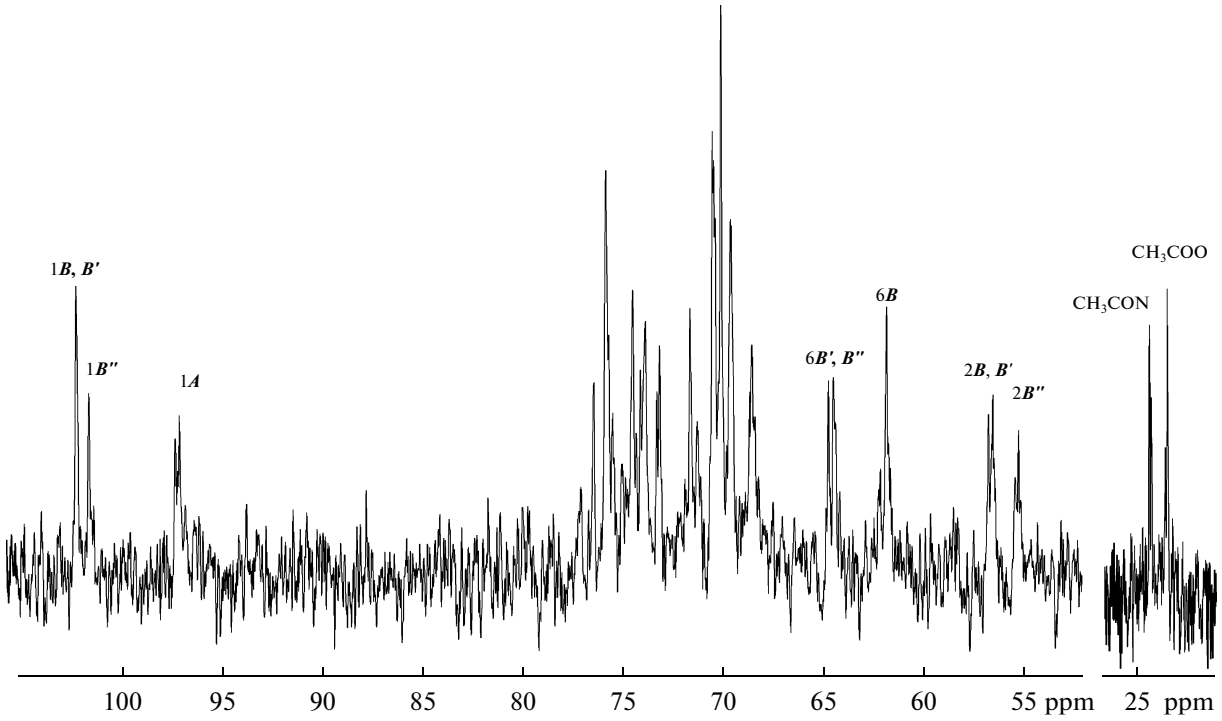

Fig. 2. Part of the ^{31}P NMR spectrum of anionic polymers of *B. subtilis* VKM B-761 cell wall. Arabic numerals indicate carbon atom positions in the residues of the disaccharide 1-phosphate polymer designated by Latin letters according to Table 4.

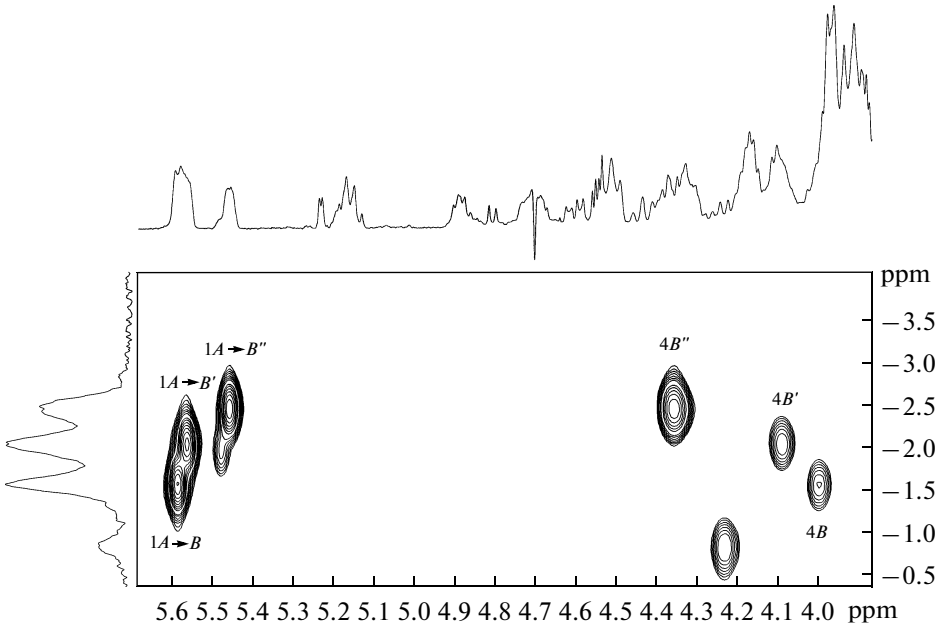

Fig. 3. Two-dimensional ^{1}H/^{13}C HMQC spectrum of the disaccharide 1-phosphate polymer of *B. subtilis* VKM B-761 cell wall. One-dimensional ^{1}H and ^{31}P spectra are located above and to the left, respectively. Arabic numerals indicate protons in the residues designated with Latin letters according to Table 4.

determined by the relative integral signal intensities of ***D***, ***D***', and ***D***'' residues. Signals in the one-dimensional ^{13}C NMR spectrum of B-761 preparation were assigned upon analyzing the heteronuclear two-dimensional ^{1}H,^{13}C gHSQC, HMQC-TOCSY, and HMBC spectra. The residue order in the sequence was

deduced from the analysis of HMBC, as well as $^{1}H,^{1}H$ ROESY and $^{1}H,^{31}P$ HMQC, spectra (Fig. 3). Correlation peaks of phosphorus and anomeric protons with the signals in the range of δ_H 5.46–5.59 (H1 of α-D-Gal*p* residues) indicated the presence of phosphate groups located at C1 hydroxyl of the C residues. Simultaneosly, correlation peaks of the same phosphorus atoms were observed with protons of C4 hydroxyls of β-D-Glc*p*NAc residues (***D***, ***D'***, and ***D''***) proving the 1,4-phosphodiester bond between residues ***C*** and ***D***. In the ROESY spectrum, correlation peaks between anomeric protons of residues ***D***, ***D'***, and ***D''*** and the protons at C6 atom (H6 and H6') in the C residues were observed, which is typical of 1 ⟶ 6 glycosidic bonds between the residues. The presence of a 1 ⟶ 6 bond between the residues was also confirmed by HMBC spectrum demonstrating C1(***D***)/H6(***C***) and H6'(***C***)/C1(***D***) correlation peaks. Therefore, the B-761 preparation contained a linear disaccharide phosphate polymer with the following structure of a partially O-acylated repeating unit:

$$\begin{array}{l} \qquad\qquad\qquad\qquad\quad (OAc)_{0.6} \\ \qquad\qquad\qquad\qquad\qquad\quad | \\ \qquad\qquad\qquad\qquad\qquad\quad 6) \\ \longrightarrow 6)\text{-}\alpha\text{-D-Gal}p\text{-}(1\text{-}P\text{-}4)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\longrightarrow \\ \qquad\qquad\qquad\qquad\qquad\quad 3) \\ \qquad\qquad\qquad\qquad\qquad\quad | \\ \qquad\qquad\qquad\qquad\quad (OAc)_{0.4} \\ \qquad\qquad\quad \boldsymbol{C} \qquad\qquad\quad \boldsymbol{D} \end{array}$$

To confirm the established structure, B-761 preparation was treated with 48% HF in order to dephosphorylate the polymer. Two disaccharides of the structure matching that of the initial polymer were isolated from the dephosphorylation products. The structure of the disaccharides was established by means of two-dimensional NMR spectroscopy (COSY, TOCSY, HSQC, and HMBC) (Table 4).

Therefore, 1,5-poly(ribitol phosphate) teichoic acid substituted with β-*D*-glucopyranose and a disaccharide 1-phosphate polymer were identified in the B-761 preparation.

DISCUSSION

The present work is part of a dedicated study of anionic polymers comprising cell walls of numerous bacilli strains of unidentified species position referred to *B. subtilis* according to a number of phenotypic characteristics. The high homology level of nucleotide sequences of 16S rRNA gene fragments does not allow correct differentiation between these strains. Specific structures of carbohydrate-containing cell wall polymers may contribute to the segregation of closely related strains into groups that can further be attributed to other species or subspecies. Earlier, we studied cell wall polymers of *B. subtilis* ssp. *subtilis* VKM B-501^{T} and *B. subtilis* VKM B-760 and VKM B-764 [12, 13]. In the present work, we report a study of a few *B. subtilis* strains (VKM). Four of the strains were received by the collection as *niger* and the other three as *aterrimus* (Table 1). The phylogenetic study confirmed that the strains belong to the *B. subtilis* group.

Polymer structure and composition were identified by chemical and NMR spectroscopic methods. This combination of techniques made it possible to establish the structure of teichoic acids, as well as to reveal the presence of two different polymers in the cell wall of strain VKM B-761.

The strains under study contained 1,5-poly(ribitol phosphate) and 1,3-poly(glycerol phosphate) teichoic acids (Table 5) glycosylated with α- and β-glucose with a low amount of alanine bound through an O-ester bond. Teichoic acids with glycosyl substitutes of either α or β-configuration of the glycosidic center are different polymers that, in the process of biosynthesis, are glycosylated by different specific glycosyl transferases [19]. It was demonstrated that glycosylation enzymes of *B. subtilis* 168 are grouped with the enzymes of teichoic acid biosynthesis and the stoichiometry of the glycoside bond may be either α or β, depending on the strain [20].

The simultaneous presence of two polymers, a teichoic acid, β-glucopyranose substituted 1,5-poly(ribitol phosphate) and a disaccharide 1-phosphate polymer with the repeating unit structure -6)-β-D-Glc*p*-(1 ⟶ 3)-α-*D*-Gal*p*NAc-(1-*P*-, was observed only in the cell wall of *B. subtilis* VKM B-761. The latter polymer and α-glucopyranose substituted 1,5-poly(ribitol phosphate) revealed in *B. subtilis* VKM B-722 and VKM B-922 were described in cell walls of gram-positive bacteria for the first time. Cell walls of strains VKM B-723 and VKM B-763 (=911) contained 1,3-poly(glycerol phosphate) substituted with β-glucopyranose. According to the modern taxonomy (StrainInfo bioportal, http://www.straininfo.net), these organisms belong to *B. atrophaeus* and, based on the data obtained, are characterized by teichoic acids of identical structures. A similar teichoic acid is present in VKM B-520; this strain is absent in other collections (Table 1).

The structures of the anionic cell wall polymers of 10 *B. subtilis* strains studied in the present and previous works [12, 13] are summarized in Table 5. The combined data make it possible to draw the following conclusions. (i) *B. subtilis* strains are heterogeneous in terms of cell wall polymer structure and composition. (ii) Based on the identical structures of teichoic acids, some strains may be joined into groups, for example, the group of strains VKM B-723 and VKM B-763 (=911) corresponds to *B. atrophaeus* species. (iii) Nine of the studied strains differ from the type strain *B. subtilis* ssp. *subtilis* VKM B-501 in polymer composition and structure. Thus, the set and structure of cell wall polymers may be an additional criterion for identifying closely related species in the *B. subtilis* group, as are

Table 5. Structures of anionic carbohydrate-containing cell wall polymers of the *B. subtilis* strain group

Strains	Teichoic acids and other phosphorus-containing polymers[1]	
B. subtilis ssp. *subtilis* VKM B-501^T	1) 1,3-poly(glycerol phosphate) with α-Glc	[12]
	2) SPP[2]: -6)-β-D-Glc*p*-(1 ⟶ 3)-α-D-Gal*p*NAc-(1-*P*-	[12]
B. subtilis VKM B-761	1) 1,5-poly(ribitol phosphate) with β-Glc	
	2) SPP: -4)-β-D-Glc*p*NAc-(1 ⟶ 6)-α-D-Gal*p*-(1-*P*-	
B. subtilis VKM B-723[3]	1,3-poly(glycerol phosphate) with β-Glc	
B. subtilis VKM B763[3]	idem	
(=VKM B-911[3])	idem	
B. subtilis VKM B-520	idem	
B. subtilis VKM B-722	1,5-poly(ribitol phosphate) with α-Glc	
B. subtilis VKM B-922	idem	
B. subtilis VKM B-760	1) 1,3-poly(glycerol phosphate);	[13]
	2) -6)-β-D-Gal*p*-(1 ⟶ 1)-sn-Gro-(1-*P*-	
	3) -6)-α-D-Glc*p*-(1 ⟶ 1)-sn-Gro-(1-*P*-	
B. subtilis VKM B-764	1) 1,3-poly(glycerol phosphate);	
	2) -6)-β-D-Gal*p*-(1 ⟶ 1)-sn-Gro-(1-*P*-	
	3) -6)-α-D-Glc*p*-(1 ⟶ 1)-[β-D-Glc*p*-(1 ⟶ 2)]-sn-Gro-(3-*P*-	[13]

Notes: [1] All polymers contained O-ester-bound D-alanine.
[2] SPP, sugar 1-phosphate polymer.
[3] The modern strain name, according to the StrainInfo bioportal (http://www.straininfo.net), is *B. atrophaeus*.

lipids, considered a reliable chemotaxonomic characteristic by a number of authors [2, 3, 21].

Taking into account the tendency in development of bacterial taxonomy toward "splitting" heterogeneous genera and species and description of taxa of a more homogenous composition, investigation of cell wall polymers in phenotypically closed strains of the genus *Bacillus* probably holds a certain promise for taxonomy.

In the future, we plan to continue the study of carbohydrate-containing cell wall polymers of bacilli, including a number of type strains of the *B. subtilis* group (*Bacillus subtilis* ssp. *inaquosorum*, *B. amyloliquefaciens, B. licheniformis, B. pumilus*, etc.).

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