ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 5, pp. 543-548. © Pleiades Publishing, Ltd., 2009.
Original Russian Text © A. S. Shashkov, N. V. Potekhina, S. N. Senchenkova, E. B. Kudryashova, 2009, published in Biokhimiya, 2009, Vol. 74, No. 5, pp. 668-674.

Anionic Polymers of the Cell Wall of *Bacillus subtilis* subsp. *subtilis* VKM B-501^T

A. S. Shashkov[1], N. V. Potekhina[2]*, S. N. Senchenkova[1], and E. B. Kudryashova[3]

[1]*Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky pr. 47, 117913 Moscow, Russia; fax: (495) 135-5328*
[2]*Faculty of Biology, Lomonosov Moscow State University, 119992 Moscow, Russia; fax: (495) 939-4309; E-mail: potekchina@hotbox.ru*
[3]*All-Russian Collection of Microorganisms (VKM), Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, 142292 Pushchino, Moscow Region, Russia; fax: (495) 923-3602*

Received March 12, 2008
Revision received November 17, 2008

Abstract—Teichoic acid and disaccharide-1-phosphate polymer were identified in the cell walls of *Bacillus subtilis* subsp. *subtilis* VKM B-501^T. The teichoic acid represents 1,3-poly(glycerol phosphate) 80% substituted by α-D-glucopyranose residues at O-2 of glycerol. The linear repeating unit of disaccharide-1-phosphate polymer contains the residues of β-D-glucopyranose, N-acetyl-α-D-galactosamine, and phosphate and has the following structure: -6)-β-D-Glc*p*-(1→3)-α-D-Gal*p*NAc-(1-*P*-. The structures of two anionic polymers were determined by chemical and NMR-spectroscopic methods. The ^{1}H- and ^{13}C-NMR spectral data on disaccharide-1-phosphate polymer are presented for the first time.

DOI: 10.1134/S0006297909050095

Key words: *Bacillus subtilis*, cell wall, teichoic acid, disaccharide-1-phosphate polymer, anionic polysaccharides, NMR spectroscopy

Teichoic acids (TA) have been studied in the cell walls of several *Bacillus subtilis* strains (168, NCTC 3610, AHU 1392, AHU 1035, AHU 1037, W23, and S31) [1-4] and in *Bacillus subtilis* var. *niger* WM [5]. The polymers are ribitol- and glycerol-containing TA and varied in sugar substituents as well as in the nature of phosphodiester bonds.

1,3-Poly(glycerol phosphate) TA substituted by α-glucosyl residues and bearing *O*-bound D-alanine residues were found in the cell wall of a typical strain of *B. subtilis* NCTC 3610 species [2]. TA with analogous structure was present in the cell wall of another strain, *B. subtilis* 168 [1], but in addition to TA also sugar-1-phosphate polymer with the disaccharide repeating unit -6)-β-D-Glc*p*-(1→3)-α-D-Gal*p*NAc-(1-*P*- was found [6]. The complete structure of the abovementioned polymers was determined by chemical and enzymatic degradation.

This paper presents the results obtained in initial studies of the content of cell walls of various strains of *B. subtilis* and some other bacilli in order to reveal correlations between strain and species differences in this group of microorganisms and the content and structure of anionic polymers of the cell wall, as was demonstrated for representatives of some genera of the order Actinomycetales [7].

The goal of the present work was to detect anionic polymers in the cell wall of *Bacillus subtilis* subsp. *subtilis* VKM B-501^T (= NCTC 3610) and to study their structures by NMR spectroscopy.

The data indicate that along with 1,3-poly(glycerol phosphate) TA with glucosyl substituents, sugar-1-phosphate polymer analogous to the strain 168 polymer is present in the cell wall of *Bacillus subtilis* subsp. *subtilis* VKM B-501^T. The structures of both polymers were supported by NMR spectroscopy.

MATERIALS AND METHODS

To obtain biomass, *B. subtilis* subsp. *subtilis* VKM B-501^T cells were grown aerobically in shaker flasks for 36 h at 28°C. The culture medium was as follows: aminopeptide, 60 ml; trypsin, 5 g; yeast extract, 1 g; soya extract, 30 ml; K_2HPO_4, 0.2 g; distilled water to 1000 ml, pH

Abbreviations: PE1, phosphoric ester; TA, teichoic acids; TSP, sodium salt of 3-(trimethylsilyl)-3,3,2,2-tetradeuteropropionic acid.

* To whom correspondence should be addressed.

before sterilization 7.2. After sterilization 50% molasses (20 ml) was added to the medium.

Cell wall preparation was obtained by cell disintegration using a UZDN-1 ultrasonic disintegrator as described in [8]. Descending paper chromatography and electrophoresis were performed using FN-3 paper from Filtrak (Germany) as described in [9].

Sugar-1-phosphate polymer was extracted from cell wall preparation (380 mg) with citrate buffer, pH 4.0 [10], and teichoic acid was further extracted with 10% TCA (1 : 10 w/v) at 4°C [9]. The extracts of carbohydrate-containing fractions were dialyzed and lyophilized (14 and 40 mg yield, respectively).

Reagents for identification of the products of degradation of teichoic acids and conditions for acidic hydrolysis of the cell wall and anionic polymers are described in [9]. Phosphoric esters were hydrolyzed using basic phosphatase (EC 3.1.3.1) from calf intestine (Sigma, USA) in ammonium acetate buffer, pH 10.4, for 2 h at 37°C. The degradation products were analyzed by high-voltage electrophoresis and paper chromatography via comparison with controls [9].

The NMR spectra were recorded for solutions in 99.96% D_2O at 30°C using a DRX-500 spectrometer from Bruker (Germany). Chemical shifts were measured with respect to the following standards: ^1H, internal TSP (sodium salt of 3-(trimethylsilyl)-3,3,2,2-tetradeuteropropionic acid), δ_H 0.0; ^{13}C, internal acetone, δ_C 31.45; ^{31}P, external (in capillary) 80% phosphoric acid, δ_P 0.0. The 2D NMR spectra were recorded according to the standard Bruker procedures. The spin lock time for ROESY was 300 msec. The $^1H/^{31}P$ HMQC spectra were optimized for spin-coupling constant $J_{H,P}$ 8 Hz.

RESULTS AND DISCUSSION

Glucose, glycerol, galactosamine, glycerol mono- and diphosphates, inorganic phosphate, and phosphoric ester (PE1) were detected in the products of acidic degradation of the cell wall of *B. subtilis* VKM B-501^T. Staining of PE1 with the Isherwood reagent suggests its sugar-phosphate nature. The data indicate that poly(glycerol phosphate) TA and another polymer in which a carbohy-

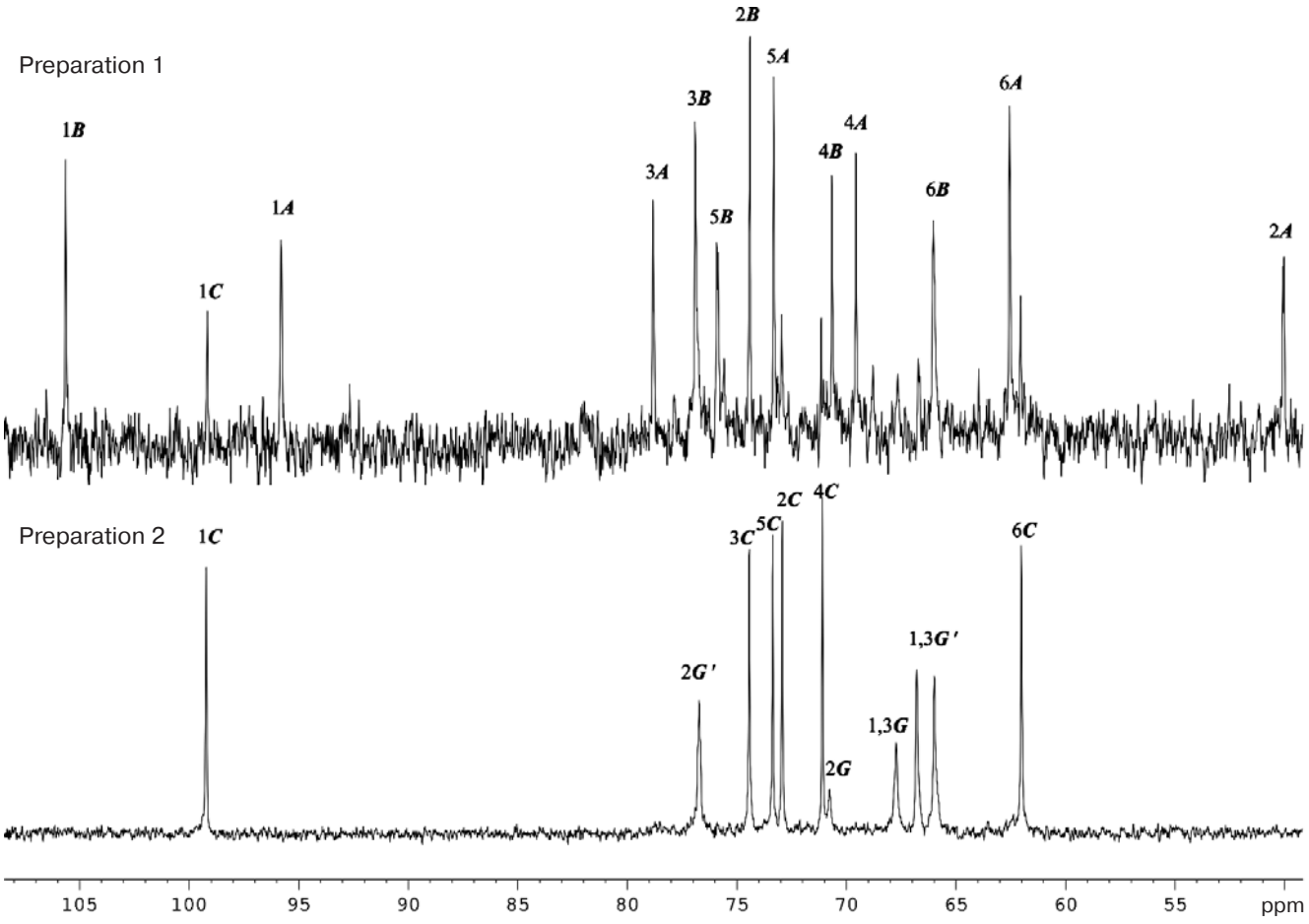

Fig. 1. ^{13}C-NMR spectra of disaccharide-1-phosphate polymer (preparation 1) and teichoic acid (preparation 2) of the cell wall of *B. subtilis* subsp. *subtilis* VKM B-501^T. Here and in Figs. 2-4 arabic digits designate atomic numbers in the residues designated in correspondence with the table.

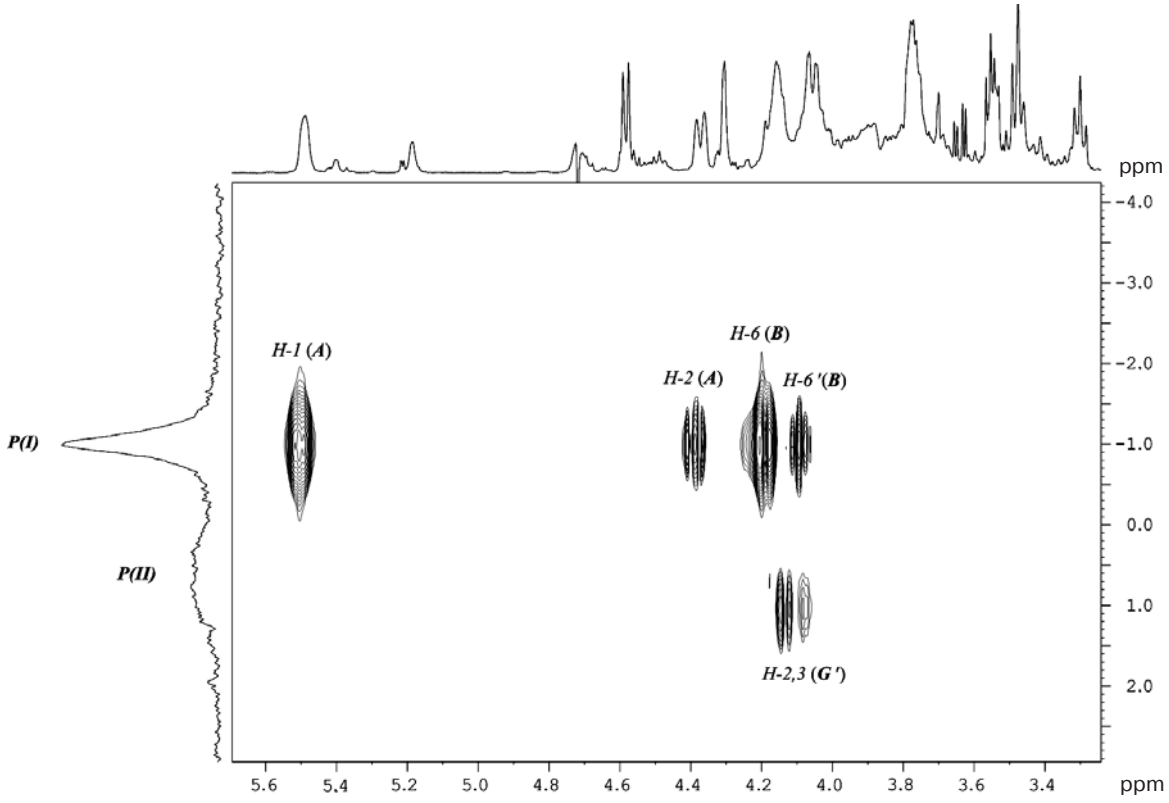

Fig. 2. $^1H/^{31}P$ HMQC spectrum of disaccharide-1-phosphate polymer (preparation 1) of the cell wall of *B. subtilis* subsp. *subtilis* VKM B-501^T. ^{31}P signals: ***P(I)***, preparation 1; ***P(II)***, preparation 2.

drate component may participate in formation of a phosphodiester bond are present in the cell wall of the studied bacillus.

Accounting for earlier data on the composition of anionic polymers of the cell walls of two *B. subtilis* strains (typical NCTC 3610 and 168) and the presence of significant amounts of galactosamine and PE1 in the acidic hydrolysate of the cell wall of the studied strain B-501 (= NCTC 3610), we suggested that sugar-1-phosphate polymer analogous to the polymer of *B. subtilis* 168 is also present. That is why we sequentially extracted the carbohydrate-containing fractions from the cell wall: with citrate buffer at pH 4.0 for sugar-1-phosphate polymer (preparation 1) and with 10% TCA for teichoic acid (preparation 2). Preparations 1 and 2 were qualitatively different.

Electrophoresis of preparation 1 revealed the presence of a major polymer with mobility m_{GroP} = 0.3. Mainly galactosamine and PE1 and also small amounts of glucose and other phosphoric esters were detected in acidic hydrolysate of this preparation.

PE1 with mobility m_{GroP} = 0.7 was stained blue-brown by the Isherwood reagent (phosphoric esters of glycerol are usually stained blue). Acidic hydrolysis (2 M HCl, 3 h, 100°C) yielded negligible amounts of inorganic phosphate, but complete degradation of this compound was not observed. Phosphomonoesterase completely hydrolyzed PE1 yielding glucose and inorganic phosphate. Chemical analysis as well as electrophoretic mobility and staining of PE1 indicate that the latter is glucose monophosphate.

Acidic hydrolysate of preparation 2 contained significant amounts of glucose, glycerol, and glycerol mono- and diphosphates, whereas PE1 and galactosamine were present in negligible amounts. Accounting for earlier data, such hydrolysate composition indicates that preparation 2 mainly consists of glucosylated poly(glycerol phosphate) TA. Electrophoresis of preparation 2 revealed the presence of one polymer with mobility m_{GroP} = 0.63. The structures of polymers in both preparations were finally identified by NMR spectroscopy.

The ^{13}C-NMR spectra of the preparations are presented in Fig. 1. As shown, they contain two different polymers (I and II) as major components: in preparation 1, polymer I prevailed and there was also an admixture of polymer II; preparation 2 consisted of almost pure polymer II. That is why in the ^{31}P-NMR spectrum of preparation 1 (Fig. 2) there were two signals at δ_P –1.0 (major) and +1.1 (minor), and in the ^{31}P-NMR spectrum of preparation 2 there was one broadened signal at δ_P +0.7. In the ^1H-NMR spectrum of preparation 1, two intense signals at δ_H 5.49 (multiplet with the broadened compo-

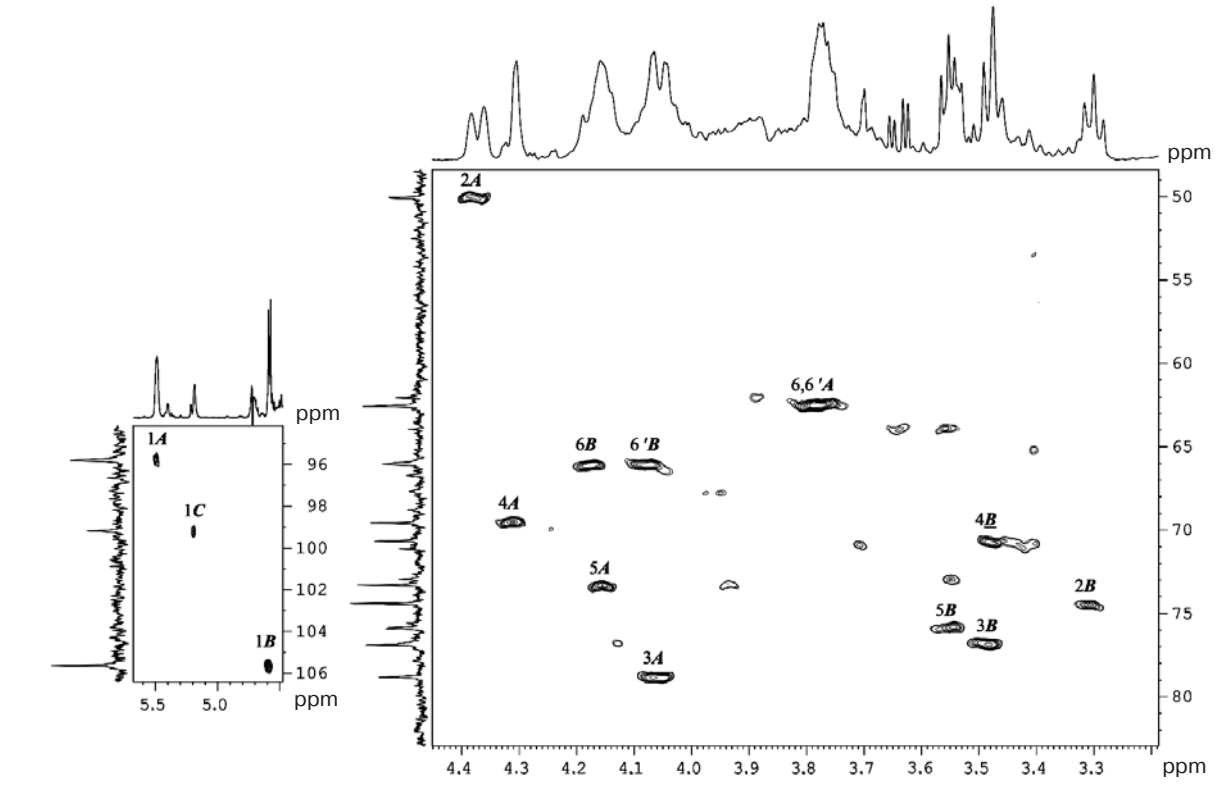

Fig. 3. 2D $^{1}H/^{13}C$ HSQC spectrum of disaccharide-1-phosphate polymer (preparation 1) of the cell wall of *B. subtilis* subsp. *subtilis* VKM B-501^{T}.

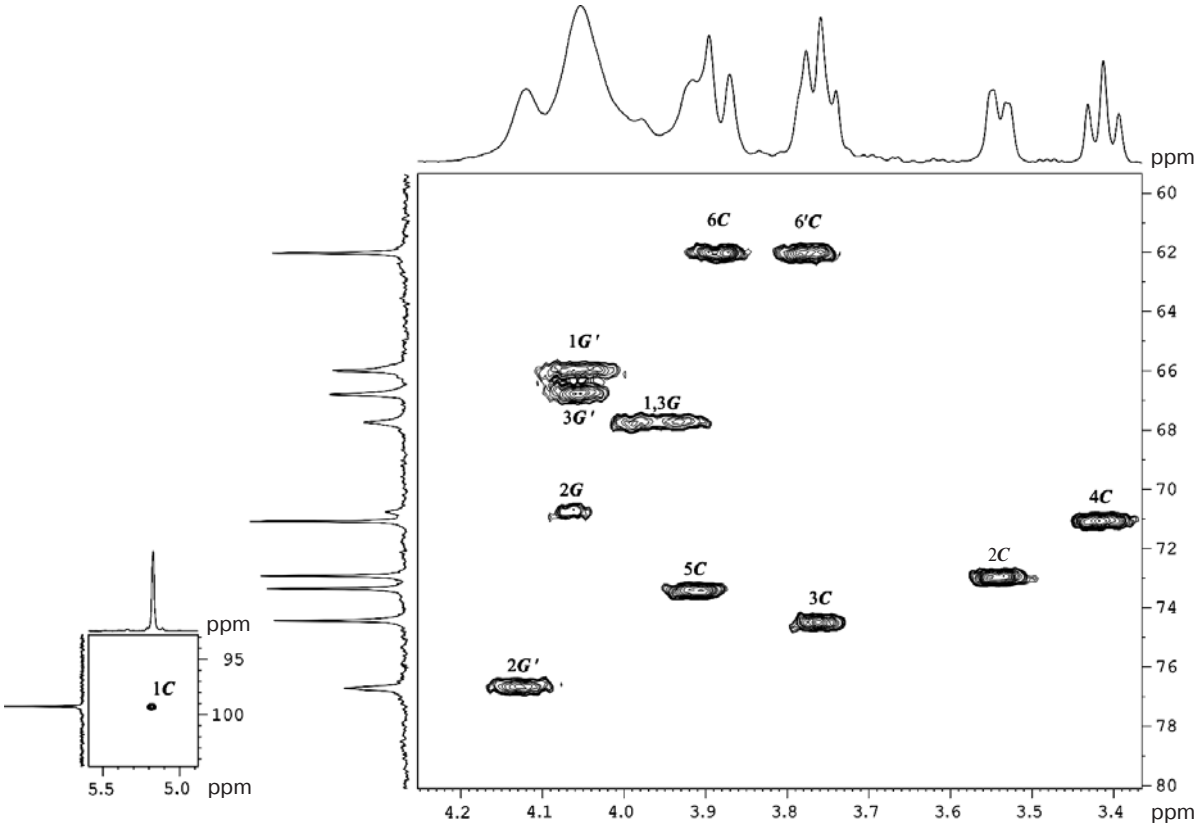

Fig. 4. 2D $^{1}H/^{13}C$ HSQC spectrum of teichoic acid (preparation 2) of the cell wall of *B. subtilis* subsp. *subtilis* VKM B-501^{T}.

nents) and 4.59 (doublet, $J_{1,2}$ 8 Hz) and a signal with significantly lower intensity at δ_H 5.17 (doublet, $J_{1,2}$ 3 Hz) in the resonance area of the protons at the anomeric carbon atoms were observed. An intense singlet at δ_H 2.05 was observed in the high-field area of the spectrum of preparation 1. One intense doublet at δ_H 5.17 (3 Hz) was observed in the low-field resonance area of the spectrum of preparation 2.

The ^{1}H- and ^{13}C-NMR spectra of preparations 1 and 2 were attributed by 2D homonuclear ^{1}H/^{1}H COSY, TOCSY, and ROESY spectra and heteronuclear ^{1}H/^{31}P HMQC and ^{1}H/^{13}C HSQC spectra. Analysis of COSY and TOCSY spectra showed that the repeating unit of the predominant polymer of preparation 1 contains 2-deoxy-2-acetamido-α-galactopyranose (α-Gal*p*NAc) and β-glucopyranose (β-Glc*p*) residues, whereas the repeating unit of polymer II consists of α-glucopyranose (α-Glc*p*) and glycerol (sn-Gro) residues. Attribution of the ^{13}C-NMR spectra by ^{1}H/^{13}C HSQC spectra (Figs. 3 and 4 and the table) and comparison of spectra of the residues with those of corresponding free pyranoses and glycerol indicate that in polymer I the α-Gal*p*NAc residue is substituted at position 3 and the β-Glc*p* residue at position 6; in polymer II the α-Glc*p* residue is terminal and the glycerol residue is substituted at all three hydroxyl groups.

Analysis of the ^{1}H/^{1}H ROESY and ^{1}H/^{31}P HMQC spectra yielded positions of glycoside and phosphodiester bonds in the polymers. An intense cross-peak H-1(***B***)/H-3(***A***) in the ROESY spectrum of polymer I indicates the presence of the 1(***B***)→3(***A***) bond. An intense cross-peak H-1(***C***)/H-2(***G′***) in the spectrum of polymer II is typical of 1→2 bond between the sugar and glycerol residues. In the ^{1}H/^{31}P HMQC spectrum of polymer I (Fig. 2) there are intense cross-peaks H-1(***A***)/*P(I)* and H-6,6′(***B***)/*P(I)* indicating the presence of phosphodiester bond between C-1(***A***) and C-6(***B***). In the ^{1}H/^{31}P HMQC spectrum of polymer II (Fig. 3) there are correlation peaks for H-1,1′,3,3′ protons of the glycerol residue and phosphorus atom *P(II)* typical of 1,3-poly(glycerol phosphate) polymers. Thus, preparation 1 mainly contains disaccharide-phosphate polymer with the following structure of the repeating unit:

(***A***) (***B***)
→3)-α-D-Gal*p*NAc-(1-*P*-6)-β-D-Glc*p*-(1→.

The relative configuration of the Gal*p*NAc residue in this polymer was established by analysis of glycosylation effects in ^{13}C-NMR spectra [11], namely by a high absolute value of α-effect for C-1 of ***B*** residue (+8.5 ppm)

Chemical shifts in ^{13}C- and ^{1}H-NMR spectra of disaccharide-1-phosphate polymer and teichoic acid of the cell wall of *B. subtilis* subsp. *subtilis* VKM B-501^T

Residue		Chemical shifts of C (δ_C acetone 31.45) and H (designated in italic; δ_H TSP 0.0) atoms					
		C-1 H-1,1'	C-2 H-2	C-3 H-3,3'	C-4 H-4	C-5 H-5	C-6 H-6,6'
Polymer I							
→3)-α-D-Gal*p*NAc-(1-*P*-	(***A***)	95.8	50.0*,**	78.8	69.6	73.3	62.6
		5.49	*4.37a*	*4.06*	*4.31*	*4.15*	*3.78*
-6)-β-D-Glc*p*-(1→	(***B***)	105.6	74.4	76.9	70.7	75.9**	66.0
		4.59	*3.31*	*3.48*	*3.48*	*3.54*	*4.17, 4.07*
Polymer II							
-1)-sn-Gro-(3-*P*-	(***G***)	67.7	70.8	67.7			
		3.99, 3.94	*4.06*	*3.99, 3.94*			
-1)-sn-Gro-(3-*P*- 2) ↑	(***G′***)	66.8	76.7	66.0			
		4.04, 4.06	*4.13*	*4.08, 4.04*			
α-D-Glc*p*-(1	(***C***)	99.2	72.9	74.4	71.1	73.3	62.0
		5.17	*3.54*	*3.76*	*3.41*	*3.91*	*3.77, 3.88*

* Signals of CH_3CON at δ_C 23.6 and 176.2 and at δ_H 2.05.
** Doublet, $^3J_{P,C}$ 7 Hz.

and by a low absolute value of the negative β-effect for C-4 of ***A*** residue (−0.3 ppm). Both effects indicate that pyranoses have equal absolute configurations, and this means D-configuration for the Gal*p*NAc residue if glucose is supposed to have the D-configuration.

The second polymer is a typical 1,3-poly(glycerol phosphate) TA in which 80% of glycerol residues are substituted by an α-D-glucopyranose residues at position 2. Possibility of the presence of two TA with glycerol residues substituted and unsubstituted at C-2 in preparation 2 is low because separation of the two polymers in an electric field by electrophoresis failed.

So, along with the earlier described teichoic acid of 1,3-poly(glycerol phosphate) nature with α-glucopyranosyl residues at O-2 of glycerol, we detected anionic polymer of sugar-1-phosphate nature in the cell wall of the studied *B. subtilis* VKM B-501^T. Sugar-1-phosphate polymer consists of linear disaccharide phosphate units with the following structure: -6)-β-D-Glc*p*-(1→3)-α-D-Gal*p*NAc-(1-*P*- and is identical to the polymer isolated from *B. subtilis* 168. The structure of this polymer is for the first time supported by NMR spectroscopy. It is interesting that a polymer with analogous structure was found earlier in the capsule of the gram-negative organism *Actinobacillus pleuropneumonia* [12]. This establishes that polymers having different localization in the surface cell layers and isolated from two organisms different in phenotype and with different Gram-staining can have the same structure.

The data indicate that two *B. subtilis* strains, VKM B-501^T studied by us and 168 described earlier [1], are identical in composition of the cell wall polymers. According to the theory of the species-specificity of teichoic acids, which we often considered earlier in relation with representatives of Actinomycetales order [7], VKM B-501^T and 168 strains are close relatives and certainly may represent the *B. subtilis* subsp. *subtilis* one species.

This work was financially supported by the Russian Foundation for Basic Research (grant No. 07-04-01054).

REFERENCES

1. Baddiley, J. (1970) *Acc. Chem. Res.*, **3**, 98-105.
2. Archibald, A. R., and Baddiley, J. (1966) *Adv. Carbohydr. Chem.*, **21**, 323-375.
3. Kaya, S., Yokoyama, K., Araki, Y., and Ito, E. (1984) *J. Bacteriol.*, **158**, 990-996.
4. Armstrong, J. J., Baddiley, J., and Buchanan, J. G. (1960) *Biochem. J.*, **76**, 610-621.
5. De Boer, W. R., Wouters, J. T. M., Anderson, A. J., and Archibald, A. R. (1978) *Eur. J. Biochem.*, **85**, 433-436.
6. Shibaev, V. N., Duckworth, M., Archibald, A. R., and Baddiley, J. (1973) *Biochem. J.*, **135**, 383-384.
7. Naumova, I. B., Shashkov, A. S., Tul'skaya, E. M., Streshinskaya, G. M., Kozlova, Y. I., Potekhina, N. V., Evtushenko, L. I., and Stackebrandt, E. (2001) *FEMS Microbiol. Rev.*, **25**, 269-284.
8. Streshinskaya, G. M., Naumova, I. B., and Panina, L. I. (1979) *Mikrobiologiya*, **48**, 814-819.
9. Shashkov, A. S., Potekhina, N. V., Evtushenko, L. I., and Naumova, I. B. (2004) *Biochemistry (Moscow)*, **69**, 658-664.
10. Pavlik, J. G., and Rogers, H. J. (1973) *Biochem. J.*, **131**, 619-621.
11. Shashkov, A. S., Lipkind, G. M., Knirel, Y. A., and Kochetkov, N. K. (1988) *Magn. Reson. Chem.*, **26**, 735-747.
12. Altman, E., Brisson, J-R., and Perry, M. B. (1988) *Biochem. Cell Biol.*, **66**, 998-1004.