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EXPERIMENTAL ARTICLES

Cell Wall Teichoic Acids of *Bacillus licheniformis* VKM B-511^T, *Bacillus pumilus* VKM B-508^T, and Other Strains Previously Assigned to *Bacillus pumilus*

G. M. Streshinskaya[a, 1], A. S. Shashkov[b], Yu. I. Kozlova[a], E. M. Tul'skaya[a], E. B. Kudryashova[c], S. N. Senchenkova[b], E. V. Ariskina[c], L. I. Evtushenko[c], and N. V. Potekhina[a]

[a] *Faculty of Biology, Moscow State University, Moscow, 119991 Russia*

[b] *Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky pr. 47, Moscow, 119991 Russia*

[c] *All-Russian Collection of Microorganisms (VKM), Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, Pushchino, Moscow oblast, 142290 Russia*

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Abstract—The teichoic acids (TAs) of type strains, viz. *Bacillus licheniformis* VKM B-511^T and *Bacillus pumilus* VKM B-508^T, as well as phylogenetically close bacteria VKM B-424, VKM B-1554, and VKM B-711 previously assigned to *Bacillus pumilus* on the basis of morphological, physiological, and biochemical properties, were investigated. Three polymers were found in the cell wall of each of the 5 strains under study. Strains VKM B-508^T, VKM B-424, and VKM B-1554 contained polymers of the same core: unsubstituted 1,3-poly(glycerol phosphate) (TA I) and 1,3-poly(glycerol phosphate) with *O*-D-Ala and N-acetyl-α-D-glucosamine substituents (TA II and TA III', respectively). The cell walls of two remaining strains contained TA I, TA II, and a poly(glycosylpolyol phosphate) with the following structure of repeating units: -6)-α-D-Glc*p*NAc(1→1)-snGro-(3-*P*-(TA III") in *"Bacillus pumilus"* VKM B-711 (100% 16S rRNA gene similarity with the type strain of *Bacillus safensis*) and -6)-α-D-Gal*p*-(1→2)-snGro-(3-*P*-(TA III"') in *Bacillus licheniformis* VKM B-511^T. The simultaneous presence of three different TAs in the cell walls was confirmed by the NMR spectroscopic DOSY methods. The structure of the polymers and localization of *O*-D-Ala residues were investigated by the chemical and NMR spectroscopic methods.

Keywords: Bacillus licheniformis, Bacillus pumilus, teichoic acid, taxonomy, NMR spectroscopy

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Members of the genus *Bacillus* are endospore-forming, rod-shaped bacteria widely occurring in soils and other habitats; some of them are known pathogens (*B. anthracis, B. cereus, B. thuringiensis*, etc.) [1]. Nonpathogenic bacteria of this group include producers of enzymes, antibiotics, and a variety of other substances of industrial importance [1]. Some strains of the genus *Bacillus* (e.g., *B. subtilis* 168 and W23) have been extensively employed as model organisms for the study of important biological problems [1].

One of the important aspects of research on bacilli, including the taxonomic issues, is the study of anionic cell surface polymers that affect many properties and vital functions of cells [2].

Recently, a number of novel species and subspecies of this genus have been described, including those phylogenetically close to *B. subtilis, B. pumilus* and *B. licheniformis* [3, 4]. Genotypic characteristics determined by the molecular methods (DNA–DNA hybridization, analysis of the *gyrB* gene encoding the B subunit of DNA gyrase, multilocus sequence analysis, etc.) and some phenotypic properties are used for differentiation of closely related species and subspecies of the *"B. subtilis"* group also traditionally including more distant species *B. licheniformis* and *B. pumilus* [3–6]. However, the difficulties in discrimination between the species at the phenotypic level have been emphasized in many works because of the high similarity of their conventional phenotypic characteristics [3, 5–7]. The usefulness of the composition and structure of cell wall teichoic acids as chemotaxonomic markers has been shown for a wide range of bacteria of the order *Actinomycetales* [8]. As far as bacilli are concerned, there are limited data on the differences between the strains of some species, including representatives of the *"B. subtilis"* group, and on the use of these data in taxonomy [9–20]. For example, the separation of *B. subtilis* into two subspecies (*B. subtilis* subsp. *subtilis* and *B. subtilis* subsp. *spizizenii*) was based on the difference between the strains in the polyols (glycerol or ribitol) as constituents of their teichoic acids, as well as on the DNA reassociation values [9]. However, at present there is no data on the composition and structure of anionic polymers of the type

[1] Corresponding author; e-mail: streshinskaya@mail.ru

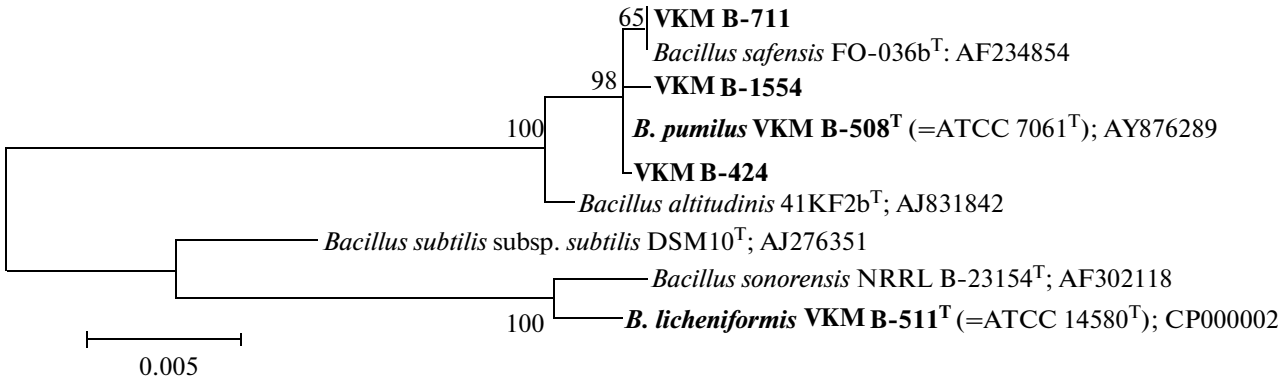

Fig. 1. Phylogenetic position of the tested strains (enhanced font) based on the analysis of the 16S rRNA gene nucleotide sequence using the neighbor-joining method. The scale shows the evolutionary distance corresponding to 5 nucleotide substitutions per every 1000 nucleotides. Numerals show the statistical reliability of the branching order determined by bootstrap analysis (the values of the bootstrap index above 50% are shown). Evolutionary distances were calculated by the Jukes–Cantor method. Phylogenetic analysis and tree construction were carried out using the MEGA5 software [21].

strains of *Bacillus* spp., except for *B. subtilis* subsp. *subtilis* VKM B-501^T [10]. All of the above emphasize the desirability of further study of the teichoic acids from bacilli for taxonomic purposes, particularly for differentiation of closely related entities (strains/species).

The goal of this work was to study the cell wall teichoic acids of the type strains of the species *B. licheniformis* and *B. pumilus* and the related bacteria previously assigned to *B. pumilus*.

MATERIALS AND METHODS

Strains. The following type strains were used in this study: *B. licheniformis* VKM B-511^T (strain) and *B. pumilus* VKM B-508^T (strain), as well as VKM B-424 (= ATCC 6635 = NBRC 12088), VKM B-711 (= NCIB 8738 = NBRC 12090), and VKM B-1554 (= ATCC 15716 = NBRC 12605). The three latter strains were initially identified as *B. pumilus* according to their morphological, physiological, and biochemical properties.

The cultures were grown aerobically at 28°C to the mid-exponential growth phase as described [10].

Analysis of 16S rRNA gene sequences. Amplification and sequencing of the 16S rRNA genes were performed as described [11]. The sequence similarities were determined using EzTaxon server (http://www.eztaxon.org). MEGA5 software was used for phylogenetic analysis and tree construction [21].

The cell walls were obtained and teichoic acid phosphorus was assayed according to [22].

Teichoic acids were extracted from lyophilized cell walls with 10% TCA (B-511^T, B-508^T, B-424, B-711, B-1554 preparations) and investigated as described [10, 22].

Paper electrophoresis and paper chromatography, as well as separation and identification of the degradation products (2 M HCl, 3 h, 100°C) of teichoic acids and cell walls, viz., monosaccharides, polyols, and phosphate esters, were performed according to [22].

NMR spectra were recorded with a Bruker Avance 600 spectrometer at 30°C for the samples in D_2O. Aqueous 80% phosphoric acid (δ_P 0.0) was used as an external reference; TSP (δ_H 0.0) and acetone (δ_C 31.45) were used as internal ones. The 2D NMR experiments were performed using the standard Bruker software. The mixing time of 150 ms and spin-lock time of 250 ms were applied in ROESY and TOCSY experiments, respectively. The $^1H/^{13}C$ and $^1H/^{31}P$ 2D HMBC experiments were optimized for the coupling constant of 5 Hz.

DOSY spectra were measured using the *stebpgp* pulse sequence, with the following parameters: Z-gradient from 2 to 95% at 512 steps, $\Delta = 0.1$ s, $\delta = 0.01$ s.

RESULTS

Phylogenetic position of the strains. The sequences of the 16S rRNA gene fragments were determined for the strains VKM B-424 (1459 bp), VKM B-711 (1467 bp) and VKM B-1554 (1437 bp). Analysis of the results confirmed affiliation of these stains with the genus *Bacillus* and showed their close phylogenetic relatedness to the species *B. pumilus* and *B. safensis* (Fig. 1). The sequence similarity between the strains VKM B-424, VKM B-711, VKM B-1554 and the type strain *B. pumilus* VKM B-508 (= ATCC 7061; sequence no. AY876289 in GeneBank) were 100, 99.9 and 99.9%, respectively (the sequences being identical or with a single-nucleotide difference). On the other hand, the 16S rRNA gene sequence of VKM B-711 was identical to that of the type strain of *B. safensis* [4].

Teichoic acids. The cell walls of the strains contained about 3% phosphorus derived from teichoic acids.

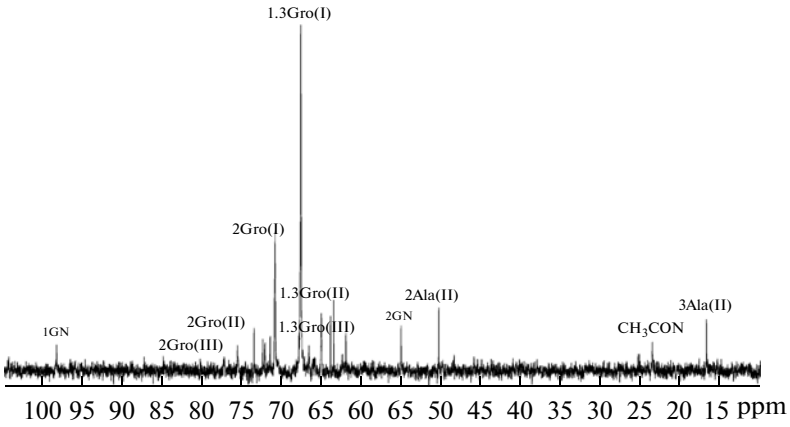

Fig. 2. The ^{13}C NMR spectrum of teichoic acids of *B. pumilus* VKM B-508^T. Arabic numerals refer to the numbers of atoms in the residues; roman numerals refer to the numbers of teichoic acids in Table 1. Abbreviations: α-Glc*p*NAc residue, GN; glycerol residue, Gro.

The acid hydrolysates of the cell walls and of the respective teichoic acid preparations from *B. pumilus* VKM B-508^T, as well as from the strains VKM B-424, VKM B-711 and VKM B-1554, yielded identical products: glucose, glucosamine, glycerol, and phosphate esters typical of glycerol teichoic acid with the 1,3-type of phosphodiester bond [23]. Electrophoresis of the B-508^T, B-424, B-711, and B-1554 preparations revealed two fractions (per each) with the mobilities m_{GroP} 1.1 and m_{GroP} 1.4.

The acid hydrolysates of the cell wall and teichoic acid preparation derived from *B. licheniformis* VKM B-511^T were found to contain galactose, glycerol, and trace amounts of glucose. The specific phosphate esters indicated the presence of glycerol teichoic acid with the 1,3-type of phosphodiester bond [23] and the possible presence of a poly(glycosylpolyol phosphate) polymer [11]. Electrophoresis of the *B. licheniformis* VKM B-511^T preparation revealed two fractions with the mobilities m_{GroP} 0.95 and m_{GroP} 1.3.

Electrophoretic separation of the teichoic acids preparations obtained from all strains under study into fractions with different mobility suggests that they contain several polymers.

NMR spectroscopy of teichoic acid preparations. The structure of the polymers including monomer composition, configurations of the glycoside centers, and positions of the phosphoester bonds were determined by 1D ^1H, ^{13}C, and ^{31}P NMR spectroscopy. The 1D NMR spectra were assigned using 2D homonuclear $^1H/^1H$ COSY, TOCSY, ROESY and heteronuclear $^1H/^{13}C$ HSQC, HMBC and $^1H/^{31}P$ HMBC experiments. Analysis of the spectra revealed the presence of 1,3-poly(glycerol phosphates) with free (TA I) or acylated (TA II) hydroxyl group at glycerol C2 in all preparations (B-511^T, B-508^T, B-424, B-711, B-1554). The acylating residue was either alanine or N-acetylalanine (Table 1). Location of the phosphate groups at the glycerol O1 and O3 was inferred from H1/P and H3/P cross-peaks in the $^1H/^{31}P$ HMBC spectra. In the ^{13}C NMR spectra of some polymers, the signals for glycerol C1 and C3 were split into doublets and the C2 signal was split into a triplet due to the $^{13}C/^{31}P$ spin–spin coupling via two and three bonds, respectively. Location of the alanyl residues at glycerol O2 was confirmed by characteristic downfield shifts of the signals for glycerol H2 (Table 1) and C2. When present, N-acetylation of the alanine residue was demonstrated by a downfield shift of the alanine H2 signal.

The preparations from all strains contained the third polymer different in structure from the teichoic acids described above.

In the B-508^T, B-424, and B-1554 preparations (Fig. 2), the third polymer (TA III') was represented by a 1,3-poly(glycerol phosphate), in which all glycerol residues (Gro) were glycosylated at O2 with a 2-acetamido-2-deoxy-α-D-glucopyranose (α-D-Glc*p*NAc) residues. The location of the sugar residue was confirmed by the presence of the correlation peaks between H1 of α-D-Glc*p*NAc and H2 and H3 of Gro in the ROESY spectra and between H1 of α-D-Glc*p*NAc and C2 of Gro in the $^1H/^{13}C$ HMBC spectra.

The third teichoic acid (TA III") in the B-711 preparation (Figs. 3 and 4) was a poly(glycosylglycerol phosphate) with the -6)-α-D-Glc*p*NAc-(1→1)-snGro-(3-*P*- repeating unit. Correlation peaks between H1 of α-D-Glc*p*NAc and H1 and H1' of Gro in the ROESY spectrum, as well as between H1 of α-D-Glc*p*NAc and C1 of Gro and between H1 and H1' of Gro and C1 of α-D-Glc*p*NAc in the HMBC spectrum proved the substitution pattern in the repeating unit. Location of the phosphate group between O6 of α-D-Glc*p*NAc and O3 of Gro was confirmed by the respective correlation peaks in the $^1H/^{31}P$ HMBC spectrum.

Table 1. The ^{1}H and ^{13}C chemical shifts in teichoic acid spectra of the cell walls of *B. licheniformis* VKM B-511^{T}, *B. pumilus* VKM B-508^{T}, B-424, B-711, and B-1554

Residue	Chemical shifts (δ_C of acetone 31.45, δ_H of TSP *0.0, italic*)					
	C1 *H1,1'*	C2 *H2*	C3 *H3,3'*	C4 *H4*	C5 *H5*	C6 *H6,6'*
Teichoic acid I (TA I)						
B. pumilus VKM B-508^{T}, B-424, B-711, B-1554; *B. licheniformis* VKM B-511^{T}						
-1)-snGro-(3-*P*-	67.8	70.9	67.8[a]			
	4.03,	*4.05*	*4.03,*			
	3.95		*3.95*			
Teichoic acid II (TA II)						
B. pumilus VKM B-508^{T}, B-424, B-711, B-1554; *B. licheniformis* VKM B-511^{T}						
-1)-snGro-(3-*P*-	65.1	75.6	65.1[b]			
2)	*4.13,*	*5.39*	*4.13*,			
|	*4.11*		*4.11*			
D-Ala-(1	17.1	51.0	175.6			
or	*1.52*	*3.97*				
|						
D-AlaNAc-(1	16.8	50.4[c]	171.5			
	1.63	*4.29*				
Teichoic acid III						
B. pumilus VKM B-508^{T}, B-424, B-1554 (TA III')						
-1)-snGro-(3-*P*-	66.7	77.3	66.0[d]			
2)	*4.03,*	*4.06*	*4.04*,			
↑	*4.00*		*4.00*			
α-D-Glc*p*NAc-(1	98.3	55.1[e]	72.4	71.5	73.5	62.0
	5.10	*3.94*	*3.81*	*3.48*	*3.94*	*3.89*, *3.80*
B. pumilus VKM B-711 (TA III")						
snGro-(3-*P*-	69.6	70.6	68.0[f]			
1)	*3.79*	*4.07*	*3.99*			
↑	*3.56*		*3.94*			
-6)-α-D-Glc*p*NAc-(1	98.8	55.0[g]	72.5	70.9	72.4	62.0[f]
	4.88	*3.97*	*3.79*	*3.59*	*3.82*	*4.14*, *4.09*
B. licheniformis VKM B-511^{T} (TA III'")						
snGro-(3-*P*-	62.7	78.8	66.0[h]			
2)	*3.80,*	*3.96*	*4.05,*			
↑	*3.78*		*3.97*			
-6)-α-D-Gal*p*-(1	99.9	69.7	70.7	70.5	71.4	66.1[d]
	5.17	*3.81*	*3.91*	*4.04*	*4.23*	*4.02*, *3.9*

Note: Phosphorus signals in the ^{31}P spectrum at δ_P [a] +0.7, [b] +0.2, [c] CH_3CON at δ_C 23.4 and 176.0 and δ_H 2.05, [d] +0.2, [e] CH_3CON at δ_C 23.6 and 176.0 and δ_H 2.08, [f] +0.6, [g] CH_3CON at δ_C 23.4 and 175.9 and δ_H 2.05, [h] +0.5.

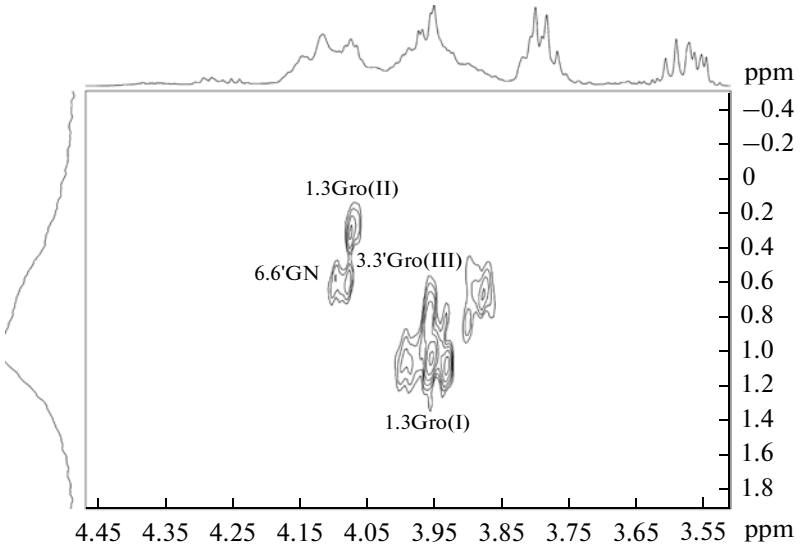

Fig. 3. The 2D heteronuclear $^{1}H/^{31}P$ HMBC spectrum of teichoic acids of *B. pumilus* VKM B-711. Arabic numerals refer to the numbers of atoms in the residues; roman numerals refer to the numbers of teichoic acids in Table 1. Abbreviations: α-Glc*p*NAc residue, GN; glycerol residue, Gro.

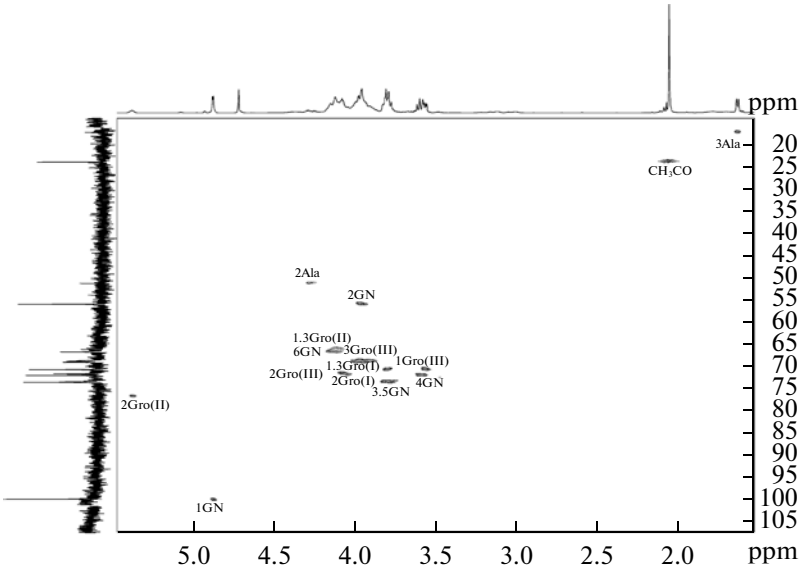

Fig. 4. The 2D heteronuclear $^{1}H/^{13}C$ HSQC spectrum of teichoic acids of *B. pumilus* VKM B-711. Arabic numerals refer to the numbers of atoms in the residues; roman numerals refer to the numbers of teichoic acids in Table 1. Abbreviations: α-Glc*p*NAc residue, GN; glycerol residue, Gro.

The structure of poly(glycosylglycerol phosphate) of the third polymer (TA III''') of the B-511^{T} preparation (Fig. 5) with the -6)-α-D-Gal*p*-(1→2)-snGro-(3-*P*- repeating unit was established in a similar way. The presence of a glycoside bond between the sugar residue and glycerol (Gro) O2 was confirmed by correlation peaks between H1 of α-D-Gal*p* and H2 of Gro in the ROESY spectrum, as well as between H1 of α-D-Gal*p* and C2 of Gro and between H2 of Gro and C1 of α-D-Gal*p* in the $^{1}H/^{13}C$ HMBC spectrum. Location of the phosphate group between O3 of Gro and O6 of α-D-Gal*p* was inferred from Gro H3,H3'/P and α-D-Gal*p* H6,H6'/P correlation peaks in the $^{1}H/^{31}P$ HMBC spectrum.

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 [2]. Some of the works devoted to the study of glycosylation enzymes demonstrated incorporation of D-hexoses into teichoic acid molecules [24–26], which corrobo-

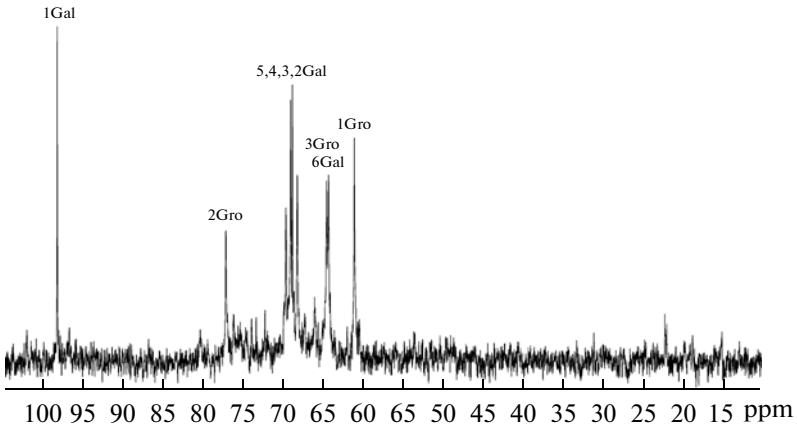

Fig. 5. The ^{13}C NMR spectrum of teichoic acids of *B. licheniformis* VKM B-511^T. Arabic numerals refer to the numbers of atoms in the residues. Abbreviations: α-Gal*p* residue, Gal; glycerol residue, Gro.

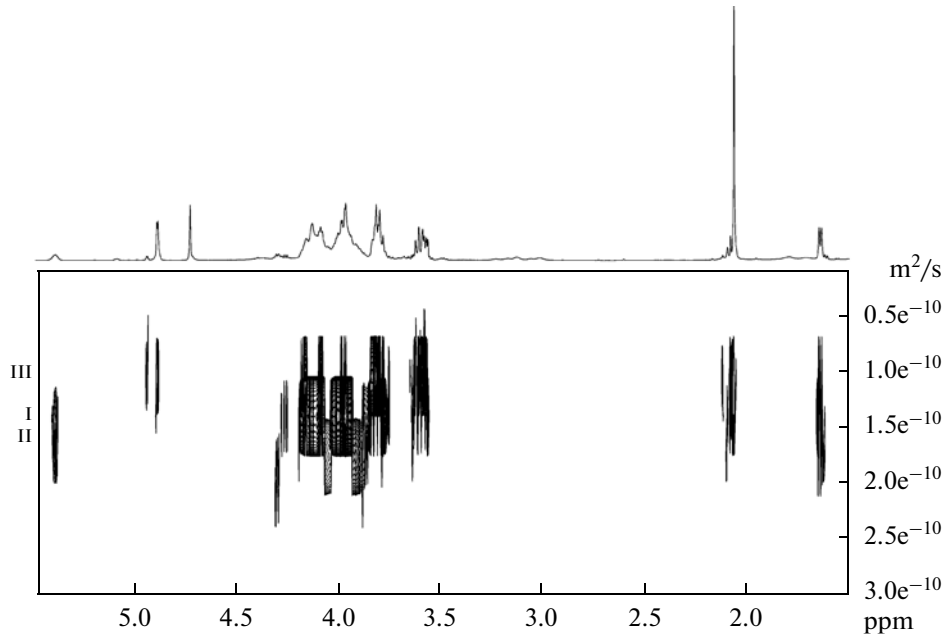

Fig. 6. The DOSY spectrum of teichoic acids of *B. pumilus* VKM B-711. Roman numerals refer to the numbers of teichoic acids; the repeating units are given in Table 1.

rates our data on determination of the absolute configuration of monosaccharides in analogous polymers [27].

It has been previously shown that the ordinary NMR spectra do not afford an opportunity to distinguish a polymer mixture from an irregular polymer [23] containing several types of monomeric units. For example, the spectra of 1,3-poly(glycerol phosphate) polymer, where the part of glycerol residues are substituted on hydroxyl group at C2 by sugar or alanine residues, are indistinguishable from the spectrum of the mixture of three respective regular polymers: 1,3-poly(glycerol phosphate) and the same polymer with sugar or alanine residues. The presence of a polymer mixture could be proved by its separation using chromatography and/or electrophoresis. However, such separation is hardly ever effective in practice [28]; these very few experiments underlay the interpretation of complex NMR spectra as the spectra of mixtures of individual polymers. Recently [29, 30], the mixtures of organic compounds, including high-molecular ones, have been analyzed by Diffusion Ordered Spectroscopy NMR (DOSY). DOSY investigation of teichoic acid preparations from bacilli showed that this technique sometimes made it possible to determine the presence of different polymers.

The DOSY spectrum of the B-711 preparation (Fig. 6) showed that the unsubstituted and alanine-

Table 2. Phosphate-containing cell wall polymers of the type strains *B. licheniformis* VKM B-511^T, *B. pumilus* VKM B-508^T, and *B. subtilis* subsp. *subtilis* VKM B-501^T

Teichoic acids and glycosyl 1-phosphate polymers (GPP)	VKM B-501^T	VKM B-508^T	VKM B-511^T
-6)-β-D-Glc*p*-(1→3)-α-D-Gal*p*NAc-(1-*P*- (GPP) [10]	◆		
1,3-poly(GroP) with α-D-Glc*p* and *O*-D-Ala [10]	◆		
1,3-poly(GroP) with α-D-Glc*p*NAc		◆	
1,3-poly(GroP) with *O*-D-Ala		◆	◆
1,3-poly(GroP)		◆	◆
-6)-α-D-Gal*p*-(1→2)-snGro-(3-*P*-			◆

substituted units of 1,3-poly(glycerol phosphate) and the units of poly(glycosylglycerol phosphate) had different diffusion coefficients D ($1.3e^{-10}$, $1.5e^{-10}$, and $1.0e^{-10}$ m^2/s, respectively) and, consequently, belonged to different polymers of different chain length and mobility.

Similarly, it was shown that the B-508^T, B-424 and B-1554 preparations contained three polymers with different diffusion coefficients D for the repeating units. However, diffusion coefficients D in the B-511^T preparation were of the same value for all units, which, nevertheless, was not an evidence of polymer heterogeneity but rather a result of accidental coincidence of mobility for the chains of different polymers. According to the spectral data, the TA I/TA II/TA III ratios in the preparations were as follows: 6 : 1 : 3 for B-511^T, 6 : 1 : 1 for B-508^T and B-424, 10 : 1 : 5 for B-1554, and 1 : 1 : 3 for B-711.

DISCUSSION

This paper presents the results of research into the cell wall teichoic acids of *B. licheniformis* VKM B-511^T and *B. pumilus* VKM B-508^T, as well as of the phylogenetically close strains (VKM B-424, VKM B-711, and VKM B-1554) initially assigned to *B. pumilus* on the basis of morphological, physiological and biochemical characteristics.

Our research showed that the type strains of the species *B. licheniformis* and *B. pumilus*, as well as the previously studied *B. subtilis* subsp. *subtilis* [10], were characterized by individual sets of cell wall anionic polymers (Table 2).

The cell walls of all strains studied in the present work contained a teichoic acid with *O*-ester-bound alanine (TA II, Table 1). As was shown in our experiments (DOSY), this teichoic acid was a separate polymer. It is known that alanine, a typical structural element of bacillary teichoic acids, modulates their functional activity associated with cation exchange and the function of bacterial autolysins. Moreover, *O*-D-alanine is a part of the regulatory mechanism used by the cell for responding to environmental changes: temperature, pH, ionic environment, particularly Mg^{+2} and NaCl concentrations, and for varying the density of negative charges of both the teichoic acid and the cell wall as a whole [2]. In the previous works [10–12], the alanine residues *O*-ester-bound to teichoic acids were revealed by the chemical methods; in the present work, localization of alanine residue on the polymer was confirmed by the methods of NMR spectroscopy. Moreover, this study showed for the first time that some of the *O*-D-alanine residues in teichoic acids of bacilli were N-acetylated (Table 1).

The cell walls of *B. pumilus* VKM B-508^T and the strains VKM B-424 and VKM B-1554 were shown to contain structurally identical teichoic acids of the 1,3-poly(glycerol phosphate) nature: unsubstituted chains (TA I) and the chains carrying *O*-D-Ala (TA II) and N-acetyl-α-D-glucosamine (TA III') residues (Tables 1, 3). Moreover, the strains *B. pumilus* VKM B-508^T and VKM B-424 with identical TA I, TA II and TA III' ratios (6 : 1 : 1) had identical 16S rRNA gene sequences. In contrast to the above-mentioned organisms, VKM B-711 contained a different type of the third polymer in its cell wall (TA III''), a poly(glycosylglycerol phosphate) with N-acetyl-α-D-glucosamine residues as components of the main chain, involved in formation of a phosphodiester bond at O6: -6)-α-D-Glc*p*NAc-(1→1)-snGro-(3-*P*-. The obtained teichoic acid ratios showed that TA III' was predominant in the cell wall of VKM B-711, while TA I was predominant in the strains VKM B-508^T, VKM B-424 and VKM B-1554.

It is worth noting that the strain VKM B-711 showed a 100% 16S rRNA gene similarity with the type strain of *B. safensis* and a 99.9% similarity with the type strain *B. pumilus* VKM B-508^T (Fig. 1). In light of our results, it may be supposed that the strain VKM B-424 is most likely a member of the species *B. pumilus*, while VKM B-711 probably belongs to another species (*B. safensis*?). The strains Sh17, Sh18, and AHU 1650 studied by other authors and described previously by the name of *B. pumilus* have a different set of polymers (Table 3) and supposedly are not members of the species *B. pumilus* either. At the same time, the presence of 1,3-poly(glycerol phosphate) and N-acetylglucosamine as an element of teichoic

Table 3. Phosphate-containing cell wall polymers of *B. pumilus* VKM B-508^T and other strains previously assigned to this species

Strain	Teichoic acids and glycosyl 1-phosphate polymers (GPP)	Reference
VKM B-508^T	1,3-poly(GroP)	p.w.
VKM B-424	1,3-poly(GroP) with *O*-D-Ala	
VKM B-1554	1,3-poly(GroP) with α-D-Glc*p*NAc	
VKM B-711	1,3-poly(GroP); 1,3-poly(GroP) with *O*-D-Ala; -6)-α-D-Glc*p*NAc-(1→2)-snGro-(3-*P*-	p.w.
Sh17	1,3-poly(GroP) with *O*-D-Ala; 1,3-poly(GroP) with α-GlcNAc*; -6)-ManNAc-(1-*P*- (GPP)	[18]
Sh18	1,5-poly(RboP); -3)-β-GlcNAc-(1→4)-Rbo-(1-*P*-*; 1,3-poly(GroP) with α-GlcNAc* and α-GalNAc*	[19]
AHU 1650	1,3-poly(GroP); -4)-GlcNAc-(1-*P*- (GPP)	[20]

Notes: glycerol phosphate, GroP; ribitol phosphate, RboP; p.w., present work.
* Polymer structures are reproduced as in original publications: without indicating the ring size of the monosaccharide constituents (*p*, pyranose) and absolute configuration of sugars.

Table 4. Teichoic acids of the cell walls of *B. licheniformis* VKM B-511^T and other strains assigned previously to this species

Strain	Set and structure of teichoic acids	Reference
VKM B-511^T	1,3-poly(GroP); 1,3-poly(GroP) with *O*-D-Ala; -6)-α-D-Gal*p*-(1→2)-snGro-(3-*P*-	p.w.
AHU 1371	-6)-α-Gal-(1→2)-Gro-(3-*P*-*	[16]
*B. licheniformis***	1,3-poly(GroP); -3)-α-D-Gal*p*-(1→1)-snGro-(3-*P*-; -3)-[β-D-Glc*p*-(1→2)]-α-D-Gal*p*-(1→1)-snGro-(3-*P*-	[15]
ATCC 9945 (= BCRC 12826 = VKM B-435)	1,3-poly(GroP); -6)-β-D-Glc*p*-(1→1)-snGro-(3-*P*-; -6)-β-D-Gal*p*-(1→1)-snGro-(3-*P*-	[13] [14] [14]
CMM 454***	1,5-poly(RboP) with β-D-Glc*p*	[17]

Notes: glycerol phosphate, GroP; ribitol phosphate, RboP; p.w., present work.
* Polymer structures are reproduced as in original publications: without indicating the ring size of the monosaccharide constituents (*p*, pyranose) and absolute configuration of sugars.
** Strain number not indicated.
*** Collection of Marine Microorganisms, Pacific Institute of Bioorganic Chemistry, Far Eastern Branch of the Russian Academy of Sciences;

acid structure in these strains may be evidence of their close genetic relationship with *B. pumilus*.

The following teichoic acids were identified in the cell wall of *B. licheniformis* VKM B-511^T: unsubstituted 1,3-poly(glycerol phosphate) (TA I) and the polymer carrying the *O*-ester-bound residues of partially N-acetylated D-alanine (TA II), as well as poly(glycosylglycerol phosphate) (TA III''') with the following structure of the repeating unit: -6)-α-D-Gal*p*-(1→2)-snGro-(3-*P*- (Table 1). The presence of several polymers in the cell walls of other strains described under the species name of *B. licheniformis* (Table 4) have been revealed previously [13–16]. These organisms differed from each other and from the type strain *B. licheniformis* VKM B-511^T in the set and structure of teichoic acids (Table 4). However, unsubstituted 1,3-poly(glycerol phosphate) and/or poly(glycosylglycerol phosphate) with galactopyra-

nose in the repeating unit was found in three out of five cases. Notably, the strains VKM B-511^T and ATCC 9945 (= BCRC 12826 = VKM B-435), for which the 16S rRNA (EF433410, EF423608) and *gyrB* (DQ309295, DQ309323) genes have been sequenced [5], are highly similar in these genes (99.6 and 95.5%, respectively).

Together with the similarity of the "classical" diagnostic characteristics, the similar set of teichoic acids and the presence of the common structural element (galactopyranose) may be evidence of close genetic relationship between some other strains of this group. On the other hand, the difference of "*B. licheniformis*" KMM 454 with 1,5-poly(ribitol phosphate) [17] from the type strain of *B. licheniformis* lacking this polymer, along with the available data on taxonomic significance of anionic polymers for other bacteria [8, 9], suggest that this strain rather represents a different species (subspecies) of bacilli.

Further studies of the strains previously assigned to the species *B. pumilus* and *B. licheniformis* on the basis of their morphological, physiological, and biochemical characteristics, using adequate taxonomic methods, will elucidate the species affiliation of the organisms considered and reveal the teichoic acids characteristic of the species (subspecies) of this group of bacilli.

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REFERENCES

1. *The Prokaryotes,* Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., and Stackebrandt, E., Eds., New York: Springer, 2006, vol. 4, pp. 530–630.
2. Neuhaus, F.C. and Baddiley, J., A Continuum of Anionic Charge: Structures and Functions of D-Alanil-Teichoic Acids in Gram-Positive Bacteria, *Microbiol. Molecul. Biol. Rev.*, 2003, vol. 67, no. 4, pp. 686–723.
3. Rooney, A.P., Price, N.P.J., Ehrhardt, C., Swezey, J.L., and Bannan, J.D., Phylogeny and Molecular Taxonomy of the *Bacillus subtilis* Species Complex and Description of *Bacillus subtilis* subsp. *inaquosorum* subsp. nov., *Int. J. Syst. Evol. Microbiol.*, 2009, vol. 59, pp. 2429–2436.
4. Satomi, M., La Duc, M.T., and Venkateswaran, K., *Bacillus safensis* sp. nov., Isolated from Spacecraft and Assembly-Facility Surfaces, *Int. J. Syst. Evol. Microbiol.,* 2006, vol. 56, no. 8, pp. 1735–1740.
5. Wang, L.-T., Lee, F.-L., Tai, C.-J., and Kasai, H., Comparison of *gyrB* Gene Sequences, 16S RNA Gene Sequences and DNA-DNA Hybridization in the *Bacillus subtilis* Group, *Int. J. Syst. Evol. Microbiol.*, 2007, vol. 57, pp. 1846–1850.
6. Roberts, M.S., Nakamura, L.R., and Cohan, F.M., *Bacillus vallismortis* sp. nov., a Close Relative of *Bacillus subtilis,* Isolated from Soil in Death Valley, California, *Int. J. Syst. Bacteriol.*, 1996, vol. 46, no. 2, pp. 470–475.
7. Fritze, D., Taxonomy of the Genus *Bacillus* and Related Genera: The Aerobic Endospore-Forming Bacteria, *Phytopathology,* 2004, vol. 94, no. 11, pp. 1245–1248.
8. Potekhina, N.V., Streshinskaya, G. M., Tul'skaya, E.M., and Shashkov, A.S., Cell Wall Teichoic Acids in the Taxonomy and Characterization of Gram-Positive Bacteria, *Methods in Microbiology,* Rainey, F.A. and Oren, A., Eds., Academic, 2012, vol. 38, part 6, pp. 131–164.
9. Nakamura, L.K., Roberts, M.S., and Cohan, F.M., Relationship of *Bacillus subtilis* Clades Associated with strains 168 and W23: A Proposal for *Bacillus subtilis* subsp. *subtilis* subsp. nov. and *Bacillus subtilis* subsp. *spizizenii* subsp. nov., *Int. J. Syst. Bacteriol.*, 1999, vol. 49, pp. 1211–1215.
10. Shashkov, A.S., Potekhina, N.V., Senchenkova, S.N., and Kudryashova, E.B., Anionic Polymers of the Cell Wall of *Bacillus subtilis* subsp. *subtilis* VKM B-501^T, *Biochemistry* (Moscow), 2009, vol. 74, no. 5, pp. 543–548.
11. Potekhina, N.V., Streshinskaya, G.M., Kozlova, Yu.I., Kudryashova, E.B, Senchenkova, S.N., Shashkov, A.S., and Anan'ina, L.N., Heterogeneous Set of Cell Wall Teichoic Acids in Strains of *Bacillus subtilis* VKM B-760 and VKM B-764, *Biochemistry* (Moscow), 2009, vol. 74, no. 12, pp. 1368–1374.
12. Streshinskaya, G.M., Shashkov, A.S., Potekhina, N.V., Kozlova, Yu.I., Tul'skaya, E.M., Senchenkova, S.N., Kudryashova, E.B., and Anan'ina, L.N., Carbohydrate-Containing Cell Wall Polymers of Some Strains of the *Bacillus subtilis* Group, *Microbiology,* 2011, vol. 80, no. 1, pp. 21–29.
13. Burger, M.M. and Glaser, L., The Synthesis of Teichoic Acids. I. Polyglycerolphosphate, *J. Biol. Chem.*, 1964, vol. 239, no. 10, pp. 3168–3177.
14. Burger, M.M. and Glaser, L., The Synthesis of Teichoic Acids. V. Polyglucosylglycerol Phosphate and Polygalactosylglycerol Phosphate, *J. Biol. Chem.,* 1966, vol. 241, no. 2, pp. 494–506.
15. Schipper, D., Structural Studies of the Teichoic Acids from *Bacillus licheniformis, Carbohydr. Res.*, 1995, vol. 279, pp. 75–82.
16. Kaya, S., Yokoyama, K., Araki, Y., and Ito, E., N-Acetylmannosaminyl(1→4)N-Acetylglucosamine, a Linkage Unit between Glycerolteichoic Acid and Peptidoglycan in Cell Walls of Several *Bacillus* Strains, *J. Bacteriol.,* 1984, vol. 158, no. 3, pp. 990–996.
17. Komandrova, N.A., Gorshkova, R.P., Isakov, V.V., Tomshich, S.V., Shevchenko, L.S., Romanenko, L.A., and Nedashkovskaya, O.I., The Structure of Teichoic Acids from Marine Microorganisms *Bacillus subtilis*

and *Bacillus licheniformis, Russ. J. Bioorg. Chem.*, 1998, vol. 24, no. 6, pp. 390–392.

18. Vann, W.F., Liu, T.Y., and Robbins, J.B., *Bacillus pumilus* Polysaccharide Cross-Reactive with Meningococcal Group A Polysaccharide, *Infect. Immun.,* 1976, vol. 13, pp. 1654–1662.
19. Kubler-Kielb, J., Coxon, B., and Schneerson, R., Chemical Structure, Conjugation, and Cross-Reactivity of *Bacillus pumilus* Sh18 Cell Wall Polysaccharide, *J. Bacteriol.,* 2004, vol. 186, no. 20, pp. 6891–6901.
20. Kojima, N., Iida, J., Araki, Y., and Ito, E., Structural Studies on the Linkage Unit between Poly(N-Acetylglucosamine1-Phosphate) and Peptidoglycan in Cell Walls of *Bacillus pumilus* AHU 1650, *Eur. J. Biochem.*, 1985, vol. 149, no. 2, pp. 331–336.
21. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S., MEGA5: Molecular Evolutionary Genetic Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods, *Mol. Biol. Evol.*, 2011, vol. 28, no. 10, pp. 2731–2739.
22. Streshinskaya, G.M., Naumova, I.B., and Panina, L.I., Chemical Composition of the Cell Wall of *Streptomyces chrysomallus,* a Producer of the Antibiotic Aurantin, *Mikrobiologiya,* 1979, vol. 48, no. 5, pp. 814–819.
23. Tul'skaya, E.M., Vylegzhanina, E.S., Streshinskaya, G.M., Shashkov, A.S., and Naumova, I.B., Heterogeneity of Glycerolteichoic Acid of the Cell Wall of *Streptomyces rutgersensis* var. *castelarense* VKM Ac-238, *Biochemistry* (Moscow)*,* 1989, vol. 54, no. 4, pp. 531–536.
24. Maino, V.C. and Young, F.E., Regulation of Glucosylation of Teichoic Acid. I. Isolation of Phosphoglucomutase in *Bacillus subtilis* 168, *J. Biol. Chem.*, 1974, vol. 249, no. 16, pp. 5169–5175.
25. Shimada, A. and Ito, E., Studies on Glucosyltransferase and Endogenous Glucosyl Acceptor in *Bacillus cereus* AHU 1030 Membranes, *J. Biochem.,* 1986, vol. 100, pp. 1507–1521.
26. Shibaev, V.N., Biosynthesis of Carbohydrate Chains of Bacterial Cell Surface, *Usp. Biol. Khim*, 1982, vol. 23, pp. 61–101.
27. Streshinskaya, G.M., Shashkov, A.S., Senchenkova, S.N., Bueva, O.V., Stupar, O.S., and Evtushenko, L.I., A Novel Teichoic Acid from the Cell Wall of *Streptomyces* sp. VKM Ac-2275, *Carbohydr. Res.,* 2007, vol. 342, pp. 659–664.
28. Potekhina, N.V., Shashkov, A.S., Terekhova, L.P., and Naumova, I.B., The Cell Wall of *Actinomadura madura* Contains Poly(galactosyl-1→2-glycerol phosphate) and Poly-(3-*O*-methylgalactosyl-1→2-glycerol phosphate), *Microbiology,* 1996, vol. 65, no. 4, pp. 458–462.
29. Viel, S., Mazarin, M., Giordanengo, R., Phan, T.N.T., Charles, L., Caldarelli, S., and Bertin, D., Improved Compositional Analysis of Block Copolymers Using Diffusion Ordered NMR Spectroscopy, *Anal. Chim. Acta*, 2009, vol. 654, pp. 45–48.
30. Balayssac, S., Trefi, S., Gilard, V., Malet-Martino, M., Martino, R., and Delsuc, M.-A., 2D and 3D DOSY ^{1}H NMR, a Useful Tool for Analysis of Complex Mixtures: Application to Herbal Drugs or Dietary Supplements for Erectile Dysfunction, *J. Pharm. Biomed. Analysis*, 2009, vol. 50, no. 4, pp. 602–612.