Microbiology, Vol. 74, No. 1, 2005, pp. 40–45. Translated from Mikrobiologiya, Vol. 74, No. 1, 2005, pp. 48–54.

EXPERIMENTAL ARTICLES

Cell Wall Teichoic Acids from *Streptomyces daghestanicus* VKM Ac-1722^T and *Streptomyces murinus* INA-00524^T

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Received July 19, 2004; in final form, September 13, 2004

Abstract—The structure of cell wall teichoic acids was studied by chemical methods and NMR spectroscopy in the type strains of two actinomycete species of the *"Streptomyces griseoviridis"* phenetic cluster: *Streptomyces daghestanicus* and *Streptomyces murinus. S. daghestanicus* VKM Ac-1722^T contained two polymers having a 1,5-poly(ribitol phosphate) structure. In one of them, the ribitol units had α-rhamnopyranose and 3-*O*-methyl-α-rhamnopyranose substituents; in the other, each ribitol unit was carrying 2,4-ketal–bound pyruvic acid. Such polymers were earlier found in the cell walls of *Streptomyces roseolus* and *Nocardiopsis albus*, respectively; however, their simultaneous presence in the cell wall has never been reported. The cell wall teichoic acid of *Streptomyces murinus* INA-00524^T was a 1,5-poly(glucosylpolyol phosphate), whose repeating unit was [-6)-β-D-glucopyranosyl-(1 ⟶ 2)-glycerol phosphate-(3-*P*-]. Such a teichoic acid was earlier found in *Spirilliplanes yamanashiensis.* The ^{13}C NMR spectrum of this polymer is presented for the first time. The results of the present investigation, together with earlier published data, show that the type strains of four species of the *"Streptomyces griseoviridis"* phenetic cluster differ in the composition and structure of their teichoic acids; thus, teichoic acids may serve as chemotaxonomic markers of the species.

Key words: Streptomyces, cell wall, teichoic acid, taxonomy, NMR spectroscopy.

The genus *Streptomyces* currently includes over 500 species, described mainly in the premolecular period of the development of taxonomy based on cultural, morphological, and physiological distinctions. Subsequent studies of DNA–DNA similarity [1–4] confirmed the validity of the existence of some species but demonstrated that some of the others do not conform to the modern concept of a bacterial species [5]. Further improvement of the taxonomic structure of the genus *Streptomyces* demands complex studies of the genomic and phenotypic characteristics of the organisms, refinement of the boundaries of the species, and revision of the species on the basis of the polyphasic approach [6].

An important task of such studies is the search for new sufficiently stable phenotypic characters that would agree with the grouping of strains performed based on genomic studies, as well as evaluation of the taxonomic significance of these characters. By the example of several streptomycete species and other organisms of the order *Actinomycetales*, it has been shown that among such characters are the structure of teichoic acids (constituting up to 60% of the dry mass in some *Streptomycetaceae* representatives [7]) and their presence in particular combinations [7–10].

The present work reports the results of our study of the cell wall teichoic acids of the type strains of the species *Streptomyces daghestanicus* and *Streptomyces murinus*, which belong to one of the phenetic species groups that were outlined within the genus *Streptomyces* in the period of active employment of numerical methods of taxonomic analysis [11].

MATERIALS AND METHODS

Strains and cultivation conditions. This work used strains *Streptomyces daghestanicus* VKM Ac-1722^T and *Streptomyces murinus* INA-00524^T, obtained from the All-Russia Collection of Microorganisms (VKM) and from the Gauze Institute of New Antibiotics (INA). The cultures were grown in flasks containing 100 ml of

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peptone–yeast extract medium on a shaker at 28°C as described earlier [9]. One-day-old cultures grown under the same conditions were used as the inoculum. The biomass for isolation of cell walls was harvested in the logarithmic growth phase (17–20 h), washed with distilled water, frozen, and stored at –20°C.

To obtain cell wall preparations, mycelium was disrupted in a 0.5% aqueous solution of SDS with the help of an ultrasonic disintegrator, precipitated by differential centrifugation [9], and lyophilized.

Isolation of the fraction of anionic polymers was performed by fractional extraction with 10% trichloroacetic acid at 4°C for 24 h, repeated two or three times. Individual polymers were separated by preparative electrophoresis [9].

Enzymatic hydrolysis of the phosphoric ester, performed to obtain glycoside, was catalyzed by phosphomonoesterase (EC 3.1.3.1; Sigma, United States) in an acetate–ammonium buffer, pH 9.8, at 37°C for 18–20 h, as described in [12].

Decomposition according to Smith, performed to reveal free glycol groups, was carried out as described in [13].

Chromatographic, physicochemical, and analytical methods used in this work were described earlier [9, 12]. Determination of the primary structure of teichoic acids by chemical methods is based on the polymer degradation by various agents (heating with acid or alkali, enzymatic hydrolysis) followed by analysis of the compounds produced and reconstruction from these data of the initial structure.

NMR spectra were recorded for 2–3% solutions of the preparations in D_2O on a DRX-500 instrument (Bruker, Germany). Acetone was used as the standard (2.225 ppm in the ^1H spectrum and 31.45 ppm in the ^{13}C spectrum). Two-dimensional spectra were taken with the use of standard methods implemented in the software provided by Bruker.

RESULTS

Streptomyces daghestanicus VKM Ac-1722^T

The cell wall of *S. daghestanicus* contained 2.5% stable phosphorus, suggesting the presence of phosphorus-containing polymers. Electrophoresis of the preparation of anionic polymers in acetate–pyridine buffer (pH 5.8) showed that it contained two compounds with different electrophoretic mobilities.

Polymer 1 ($m_{Gro\mathit{P}}$ 0.8). In acidic hydrolysate (2 M HCl, 100°C, 3 h) of the polymer, we found rhamnose, ribitol mono- and diphosphates, anhydroribitol phosphate, ribitol, anhydroribitol, inorganic phosphate, and a small amount of a monosaccharide whose location upon chromatography in a pyridine–benzene–butane-1-ol–water (3 : 1 : 5 : 3, vol/vol) system corresponded to that of 3-*O*-methylrhamnose. Detection of ribitol mono- and diphosphates among the hydrolysis products testifies to the presence of a poly(ribitol phosphate) chain.

Hydrolysis of the polymer with alkali (1 M NaOH, 100°C, 3 h) revealed a phosphoric ester whose subsequent acidic hydrolysis showed it to contain ribitol monophosphate and rhamnose, suggesting that the phosphate residue is bound to ribitol rather than to rhamnose. Treatment of the phosphoric ester with phosphomonoesterase resulted in the formation of a glycoside that was similar in its chromatographic mobility to rhamnosylribitol, which had been characterized earlier [14]. These results indicate that the phosphoric ester is rhamnosylribitol phosphate.

In the ^1H spectrum of the polymer, peaks characteristic of a rhamnopyranose residue could be seen. In the region of the anomeric proton resonance, there occurred, in addition to the major peak of the rhamnose H-1 (5.09 ppm), a minor peak with a chemical shift of 5.13 ppm. A singlet in the *O*-methyl group resonance region (3.45 ppm) was easily discernable, suggesting methylation of a part of rhamnose residues. The identity of the subspectrum of this component to the spectrum of 3-*O*-methylrhamnose of the *Streptomyces roseolus* ISP 5174 teichoic acid [14] suggests that it is most probably 3-*O*-methylrhamnose. This conclusion is supported by data from the analysis of the ^{13}C NMR spectrum (Fig. 1, Table 1). This spectrum exhibited 11 major peaks: one peak of double integral intensity (71.35 ppm) and several more peaks, of which some can be identified as representing the 3-*O*-methylrhamnose residue and others as belonging to unsubstituted ribitol residues. The chemical shifts belonging to the dominant ribitol residues are typical of 1,5-poly(ribitol phosphate) carrying glycosyl substituents at the C-4(2) positions.

Thus, polymer 1 is a 1,5-poly(ribitol phosphate) in which most ribitol phosphate residues are substituted with α-rhamnosyl residues and some are substituted with 3-*O*-methyl-α-rhamnosyl residues. The molecular mass of the polymer (13360 Da) suggests the presence of about 35 repeating units.

Polymer 2 ($m_{Gro\mathit{P}}$ 1.5). Greater mobility in the electric field testifies to greater density of the electric charge in polymer 2. Among the products of its acidic hydrolysis, we identified ribitol mono- and diphosphates, ribitol, inorganic phosphate, and pyruvic acid. Glycosyl substituents were not revealed. The polymer was not destroyed under conditions of alkaline hydrolysis (1 M NaOH, 100°C, 3 h) or periodate oxidation.

As in the case of polymer 1, ribitol mono- and diphosphates were formed during acidic hydrolysis, indicating the presence of a poly(ribitol phosphate) chain, whereas stability of the polymer during heating with alkali testified to the absence of free hydroxyl groups neighboring phosphodiester groups [15], and resistance to periodate oxidation followed by reduction with $NaBH_4$ (decomposition according to Smith) indicated a lack of free glycol groups. Based on the above

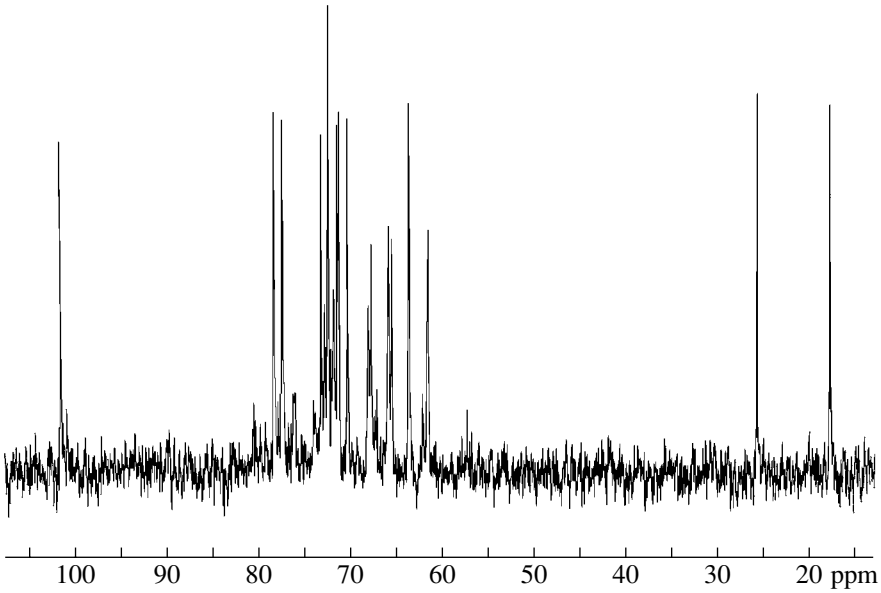

Fig. 1. ^{13}C NMR spectrum of the teichoic acids of *S. daghestanicus* VKM Ac-1722^{T}.

data, we arrived at a preliminary conclusion about possible substitution of ribitol phosphate residues with ketal-bound pyruvic acid, as had been established for an analogous polymer of *Nocardiopsis albus* DSM 43120 [7]. NMR spectroscopy of the polymer showed that its ^{13}C NMR spectrum (Fig. 1; Table 1) contained six major signals, of which two had double integral intensity and were widened or split due to the interaction with phosphorus atoms. Three signals—at 25.8 ppm (CH_3), 102.1 ppm (quaternary carbon atom), and 176.6 ppm (CO)—were identified as representing a pyruvic acid residue in a six-atom cycle [16].

Thus, for the five carbon atoms of the ribitol residue, there remained three unassigned signals, including two of the above-mentioned signals of double integral intensity. This suggested symmetric substitution of ribitol residues with pyruvate. The widening of the signal at 65.45 ppm (CH_2-group) and the splitting of the signal at 76.6 ppm suggested the presence of a 1,5-poly(ribitol phosphate) chain. The molecular mass of the polymer was 9900 Da, which corresponds to about 35 repeating units.

Thus, the second teichoic acid of *S. daghestanicus* VKM Ac-1722^{T} is also a 1,5-poly(ribitol phosphate), which, however, contains 2,4-ketal–bound pyruvic acid as a substituent.

Streptomyces murinus INA-00524^{T}

The cell wall of this strain contained about 1% teichoic acids. Two predominant compounds, glucose and glycerol monophosphate, were identified in acidic

Table 1. Chemical shifts (δ, ppm) of carbon atoms in the ^{13}C NMR spectrum of the teichoic acids of *S. daghestanicus* VKM Ac-1722^{T}

Polymer	Fragment	C-1	C-2	C-3	C-4	C-5	C-6	OCH3
1	-1)-Rib-ol-(5-*P*- 4) ↑	67.90	71.35	72.50	78.40	65.70		
	α-Rha*p*-(1	101.60	71.55	71.35	73.30	70.50	18.0	
1	-1)-Rib-ol-(5-*P*- 4) ↑	67.90	71.35	72.50	78.40	65.70		
	α-Rha*p*3Me-(1	101.7	67.80	80.65	72.15	70.25	18.0	57.40
2	-1)-Rib-ol-(5-*P*- 2 4 Pyr	66.0 176.6	77.5 102.1	61.7 25.8	77.5	66.0		

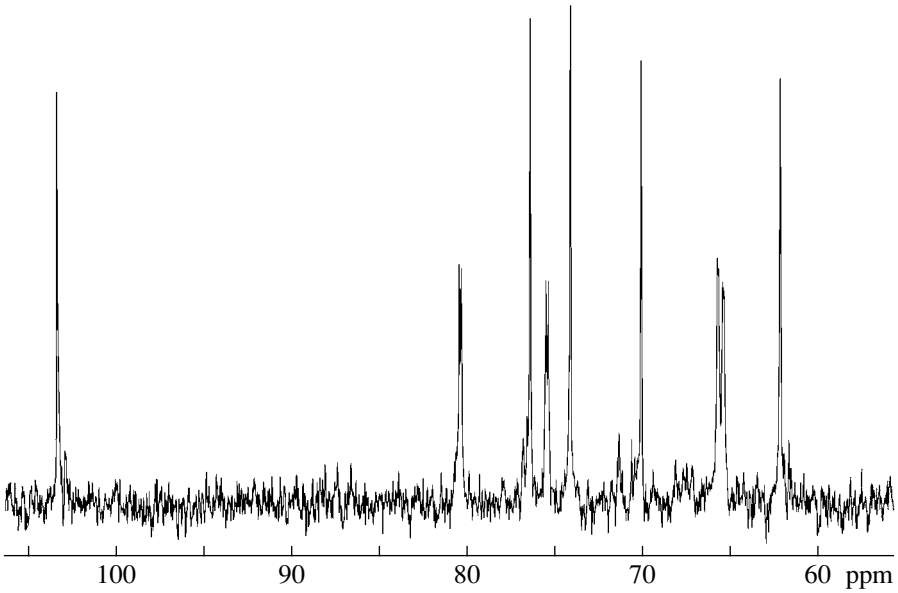

Fig. 2. ^{13}C NMR spectrum of the teichoic acid of *S. murinus* INA-00524^{T}.

hydrolysates of both the cell wall and the isolated polymer. This fact, together with the absence of glycerol diphosphate from the acidic hydrolysates, allowed us to suggest that the polymer is a poly(glycosylglycerol phosphate), i.e., a teichoic acid in which OH-groups of both glycerol and the saccharide component are involved in the formation of phosphodiester bonds between repeating units. In such polymers, the glycoside bond occurs at the O-1 or O-2 atom of glycerol, whereas the phosphodiester bond is always at the O-6 atom of glucose.

Subsequent study of the polymer by NMR spectroscopy showed that the ^{13}C spectrum of the teichoic acid (Fig. 2, Table 2) contained nine signals, of which one occurred in the resonance region of anomeric carbon atoms of sugars. Two signals, at 80.7 and 75.7 ppm, were split into doublets, and two others, at 66.0 and 65.7 ppm, were widened due to spin–spin interactions with a phosphorus atom. Analysis of the chemical shifts in the ^{13}C spectrum showed the identity of the polymer studied with an earlier investigated teichoic acid from the cell wall of *Spirilliplanes yamanashiensis* VKM Ac-1993, which is a poly(glucosylglycerol phosphate) of the following structure: poly[-6)-β-D-glucopyranosyl-(1 ⟶ 2)-glycerol phosphate-(3-*P*-] [17]. The molecular mass (6634 Da) of the polymer corresponded to 21 repeating units.

DISCUSSION

The type strains of the species *S. daghestanicus* (VKM Ac-1722^{T}) and *S. murinus* (INA-00524^{T}) studied in this work and the earlier studied [18] type strains of the species *S. griseoviridis* (VKM Ac-622^{T}) and *S. chryseus* (VKM Ac-200^{T}), belonging to the *"Streptomyces griseoviridis"* phenetic cluster [11], proved to differ in the composition and structure of the anionic cell wall polymers. *S. daghestanicus* contains, in equal amounts, two teichoic acids with 1,5-poly(ribitol phosphate) chains; one of them has α-rhamnose and 3-*O*-methyl-α-rhamnose as substituents, and in the other one the substituent is pyruvic acid. The simultaneous presence of two such polymers in the cell wall of one organism has never been reported before. *S. murinus* contains a poly(glucosylglycerol phosphate) of the following structure: [-6)-β-D-glucopyranosyl-(1 ⟶ 2)-glycerol phosphate-(3-*P*-]. In *S. griseoviridis*, a poly(glycosylglycerol phosphate) was revealed whose

Table 2. Chemical shifts (δ, ppm) of carbon atoms in the ^{13}C NMR spectrum of the teichoic acid of *S. murinus* INA-00524^{T}

Fragment	C-1	C-2	C-3	C-4	C-5	C-6
Gro-(3-*P*-2) ↑ -6)-β-Glc*p*-(1	62.3 103.4	80.4 74.2	65.5 76.5	 70.2	 75.5	 65.8

Table 3. Repeating units of teichoic acids and products of acidic degradation of cell walls of organisms belonging to the "*Streptomyces griseoviridis*" phenetic cluster

Teichoic acid monomer and some products of cell wall acidic hydrolysis (2 M HCl, 100°C, 3 h)	*S. daghestanicus* VKM Ac-1722^T	*S. murinus* INA-00524^T	*S. griseoviridis* VKM Ac-622^T [18]	*S. chryseus* VKM Ac-200^T [18]
Teichoic acid monomer	I. 1)-Rib-ol[1]-(5-*P*- 4(2) ↑ Rha/Rha3Me[2]	-6)-β-D-Glc*p*[4]- (1 ⟶ 2)-Gro[5]-(3-*P*-	-6)-α-D-Gal*p*[6]- (1 ⟶ 3)- Gal*p*NAc[7]- (1 ⟶ 1)-Gro-(3- *P*-	I. –1)-Gro-(3-*P*-
	II. 1)-Rib-ol-(5-*P*- 4 2 \ / Pyr[3]			II. 1)-Rib-ol-(5-*P*- 4(2) ↑ GlcNAc[8]
Glucose		+		
Galactose			+	
Rhamnose	+			
3-*O*-methylrhamnose	+			
Glycerol		+	+	+
Ribitol	+			+
Pyruvic acid	+			

[1] Ribitol; [2] rhamnose/methylrhamnose; [3] pyruvic acid; [4] glucopyranose; [5] glycerol; [6] galactopyranose; [7] 2-acetamido-2-deoxy-β-D-galactopyranose; [8] 2-acetamido-2-deoxy-β-D-glucopyranose.

repeating unit was [-6)-α-D-galactopyranosyl-(1 ⟶ 1)-(1 ⟶ 3)-2-acetamido-2deoxy-β-D-galactopyrosyl-(1 ⟶ 1)(glycerol-(3-*P*-] [18]. *S. chryseus* contains two teichoic acids: 1,3-poly(glycerol phosphate) and 1,5-poly(ribitol phosphate) substituted with 2-acetamido-2-deoxy-β-D-glucopyranosyl residues at position 4(2) of ribitol [18].

The differences revealed in the composition and structure of the cell wall teichoic acids of the aforementioned strains show that these polymers and their structural components can be used as species-specific chemotaxonomic markers of the members of the *"Streptomyces griseoviridis"* phenetic cluster. It should be noted that the status of *S. griseoviridis, S. chryseus*, and *S. murinus* as independent species has been confirmed by DNA–DNA hybridization of their type strains [4].

Table 3 shows the repeating units of the teichoic acids of the strains studied, as well as some products of acidic hydrolysis of their cell walls. These products are easily detectable by common chromatographic methods and may be widely used, along with other phenotypic characters, for identification of newly isolated *Streptomyces* strains.

It is noteworthy that one of the two teichoic acids found in *S. daghestanicus*, namely, the 1,5-poly(ribitol phosphate) with rhamnosyl and 3-*O*-methylrhamnosyl residues, was earlier revealed in the *Streptomyces roseolus* cell wall, but as a single anionic polymer [14], whereas the other teichoic acid, the pyruvylated 1,5-poly(ribitol phosphate), was earlier found in *Nocardiopsis albus*; the latter organism contained two more teichoic acids in the cell wall [7]. The teichoic acid revealed in the cell wall of *S. murinus* was earlier found in *Spirilliplanes yamanashiensis* VKM Ac-1993^T, along with three other anionic cell wall polymers [17]. In the present work, we studied the structure of this teichoic acid in more detail we present its ^{13}C spectrum in Fig. 2.

The presence of two or more teichoic acids with different anionic properties is a fact attracting attention since they are localized and perform their functions in the cell wall, on the surface of which zones with different density of negative charge may occur [19] and autolytic enzymes (usually, positively charged proteins) are concentrated [20].

ACKNOWLEDGMENTS

The authors are indebted to the late Prof. Irina Borisovna Naumova (1931–2003), who was the leader of the team that performed this work.

This work was supported in part by INTAS (Brussels, Belgium, grant no. 01-2040) Russian Foundation for Basic Research (grant no. 04-04-49096), and the program "Leading Scientific Schools of the Russian Federation" (grant no. 1557 2003 3).

REFERENCES

1. Labeda, D.P. and Lyons, A.J., Deoxyribonucleic Acid Relatedness among Species of the "*Streptomyces cya-*

neus" Cluster, *Syst. Appl. Microbiol*, 1991, vol. 14, no. 1, pp. 158–164.

2. Labeda, D.P., DNA Relatedness among Strains of the *Streptomyces lavendulae* Phenotypic Cluster Group, *Int. J. Syst. Bacteriol.*, 1993, vol. 43, no. 3, pp. 822–825.
3. Labeda, D.P., DNA Relatedness among Verticil-Forming *Streptomyces* Species (Formerly *Streptoverticillium* Species), *Int. J. Syst. Bacteriol.*, 1996, vol. 46, no. 3, pp. 699–703.
4. Labeda, D.P., DNA Relatedness among the *Streptomyces fulvissimus* and *Streptomyces griseoviridis* Phenotypic Cluster Group, *Int. J. Syst. Bacteriol.*, 1998, vol. 48, no. 3, pp. 829–832.
5. Wayne, I.G., Brenner, L.J., Colwell, R.R., *et al.*, International Committee on Systematic Bacteriology. Report of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics, *Int. J. Syst. Bacteriol.,* 1987, vol. 37, no. 1, pp. 463–464.
6. Anderson, A.S. and Wellington, E.M.H., The Taxonomy of *Streptomyces* and Related Genera, *Int. J. Syst. Evol. Microbiol.*, 2001, vol. 51, no. 3, pp. 797–814.
7. Naumova, I.B., Shashkov, A.S., Tul'skaya, E.M., Streshinskaya, G.M., Kozlova, Yu.I., Potekhina, N.V., Evtushenko, L.I., and Stackebrandt, E., Cell Wall Teichoic Acids: Structural Diversity, Species Specificity in the Genus *Nocardiopsis*, and Chemotaxonomic Perspective, *FEMS Microbiol Rev.*, 2001, vol. 25, no. 3, pp. 269–283.
8. Potekhina, N.V., Tul'skaya, E.M., Shashkov, A.S., Taran, V.V., Evtushenko, L.I., and Naumova, I.B., Species Specificity of Teichoic Acids in the Actinomycete Genus *Glycomyces, Mikrobiologiya*, 1998, vol. 67, no. 3, pp. 414–418.
9. Streshinskaya, G.M., Kozlova, Yu.I., Shashkov, A.S., Evtushenko, L.I., and Naumova, I.B., The Cell Wall Teichoic Acids of Streptomycetes from the *"Streptomyces cyaneus"* Cluster, *Mikrobiologiya*, 2003, vol. 72, no. 4, pp. 510–515.
10. Kozlova, Yu.I., Shashkov, A.S., Evtushenko, L.I., and Naumova, I.B., Anionic Carbohydrate-Containing Polymers of the Cell Walls of Two Genospecies of *Streptoverticillium, Biokhimiya*, 1999, vol. 64, no. 6, pp. 805–812.
11. Williams, S.T., Goodfellow, M., Alderson, G., Wellington, E.M.H., Sneath, P.H.A., and Sackin, M.J., Numerical Classification of *Streptomyces* and Related Genera, *J. Gen. Microbiol.*, 1983, vol. 129, no. 1/2, p. 1743.
12. Streshinskaya, G.M., Naumova, I.B., Romanov, V.V., and Shashkov, A.S., The Structure of the Ribitol-Teichoic Acid of the Cell Wall of *Streptomyces azureus* RIA 1009, *Bioorg. Khim.*, 1981, vol. 7, no. 9, pp. 1409–1418.
13. Naumova, I.B. and Belozerskii, A.N., Ribitol-Teichoic Acid from the Cell Wall of an Actinomycete Producing the Antibiotic Aurantin, *Biokhimiya*, 1966, vol. 31, no. 6, pp. 1276–1282.
14. Potekhina, N.V., Shashkov, A.S., and Kuznetsov, V.D., 3-*O*-Methyl Rhamnose in the Cell Wall Teichoic Acid of *Streptomyces roseolus* ISP 5174, *Biokhimiya*, 1992, vol. 57, no. 8, pp. 1206–1214.
15. Kelemen, M.V. and Baddeley, J., Structure of the Intracellular Glycerol Teichoic Acid from *Lactobacillus casei* ATCC 7469, *Biochem. J.*, 1961, vol. 80, pp. 246–254.
16. Thurow, H., Choy, Y.M., Frank, N., and Niemann, H., The Structure of *Klebsiella* Serotype 11 Capsular Polysaccharide, *Carbohydr. Res*, 1975, vol. 41, pp. 241–255.
17. Shashkov, A.S., Streshinskaya, G.M., Evtushenko, L.I., and Naumova, I.B., NMR-Based Identification of Cell Wall Anionic Polymers of *Spirilliplanes yamanashiensis* VKM Ac-1993^T, *Carbohydr. Res.*, 2001, vol. 336, no. 2, pp. 237–242.
18. Kozlova, Yu.I., Streshinskaya, G.M., Shashkov, A.S., Evtushenko, L.I., Gavrish, E.Yu., and Naumova, I.B., The Structure of Anionic Carbohydrate-Containing Polymers of the Cell Walls of Some Representatives of the Order *Actinomycetales, Biokhimiya*, 2000, vol. 65, no. 10, pp. 1432–1439.
19. Seltmann, G. and Holst, O., *The Bacterial Cell Wall*, Berlin: Springer, 2002, pp. 139–140.
20. Wecke, J., Perego, M., and Fischer, W., D-Alanine Deprivation of *Bacillus subtilis* Teichoic Acids Is Without Effect on Cell Growth and Morphology but Affects the Autolytic Activity, *Microb. Drug Resist.*, 1996, vol. 2, no. 1, pp. 123–129.