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EXPERIMENTAL ARTICLES

Anionic Carbohydrate-Containing Cell Wall Polymers of *Streptomyces melanosporofaciens* and Related Species

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Abstract—The structures of cell wall anionic carbohydrate-containing polymers in *Streptomyces melanosporofaciens* VKM Ac-1864^T and phylogenetically close organisms—*S. hygroscopicus* subsp. *hygroscopicus* VKM Ac-831^T, *S. violaceusniger* VKM Ac-583^T, *S. endus* VKM Ac-1331^T, *S. endus* VKM Ac-129, and *S. rutgersensis* subsp. *castelarensis* VKM Ac-832^T—have been comparatively studied by chemical and NMR spectroscopic methods. The natural polymer of a new, previously unknown structure, Kdn (3-deoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid) with β-galactose residues at C-9, has been found in the cell walls of all the strains under study. The cell walls of all the studied organisms contain three teichoic acids (TA): a predominant TA (1,3-poly(glycerol phosphate) with N-acetylated α-glucosaminyl substitutes by C-2 of glycerol, and minor TAs, 1,3- and 2,3-poly(glycerol phosphate) polymers without substitution. Their chains have *O*-acetyl and *O*-lysyl groups. Microorganisms of the above-mentioned species differ in the number of α-glucosaminyl substitutes and in the degree of their acetylation in the predominant teichoic acid.

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

Anionic carbohydrate-containing polymers (ACP) of the bacterial cell wall have vital functions, and the interest in the study of these compounds has not subsided over many years [1–3]. The study of the chemical composition of ACP in microorganisms not yet investigated in this respect provides insight into the diversity of natural biopolymers and may be useful for taxonomical purposes, in particular, for species differentiation [4]. Previously, we have studied the cell wall teichoic acids of representatives of phylogenetically and phenotypically close species of the genus *Streptomyces*: *S. hygroscopicus, S. endus*, and *S. violaceusniger* [5], comprising the phenetic cluster *"Streptomyces violaceusniger"* [6, 7]; and *S. rutgersensis* subsp. *castelarensis* VKM Ac-832^T, which has a similar set of teichoic acids [8] and is most closely related to *S. melanosporofaciens* according to 16S rDNA nucleotide sequences (99.7% similarity; calculated by the sequences deposited in the GenBank database, NCBI) [9]. All these organisms were shown to possess similar sets of glycerophosphate teichoic acids: predominant 1,3-poly(glycerol phosphate) with partially N-acetylated irregular α-glucosaminyl substitutes, and minor quantities of 1,3- and 2,3-poly(glycerol phosphate) polymers without substitutes.

The goal of the present work was to determine the structures of cell wall anionic carbohydrate-containing polymers in *S. melanosporofaciens* VKM Ac-1864^T, one more representative of the above-mentioned cluster, and to compare them with the cell wall polymers of the above-mentioned organisms.

MATERIALS AND METHODS

Strains. The type strains *S. melanosporofaciens* VKM Ac-1864^T (=ISP 5318^T), *S. hygroscopicus* subsp. *hygroscopicus* VKM Ac-831^T (=ISP 5578^T), *S. violaceusniger* VKM Ac-583^T (=ISP 5563^T), *S. rutgersensis* subsp. *castelarensis* VKM Ac-832^T (=ATCC 15191^T), and *S. endus* VKM Ac-1331^T (=ISP 5187^T), as well as *S. endus* VKM Ac-129, were used in the work.

Cultivation of microorganisms, obtaining of cell walls, isolation and chemical analysis of polymers were carried out as described previously [8, 10]. Electrophoresis of the phosphorus-containing preparation was performed in pyridine–acetate buffer, pH 5.6 (A). The following solvent systems were used for descend-

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ing paper chromatography: pyridine–benzene–butane-1-ol–water (3 : 1 : 5 : 3, vol/vol) (B), pyridine–ethylacetate–acetic acid–water (5 : 5 : 1 : 3, vol/vol) (C), and butane-1-ol–acetic acid–water (5 : 1 : 4, v/v) (D) [5, 8].

Reagents used for detection of compounds: Isherwood's reagent for teichoic acids and their degradation products; ninhydrin for amino sugars; 5% $AgNO_3$ in aqueous ammonia for glycerol, glycosides, and monosaccharides; and aniline phthalate for monosaccharides.

NMR spectra were recorded with a DRX-500 (Bruker, Germany) spectrometer for 2–3% solutions in D_2O at 30°C with acetone (ΔH 2.225, ΔC 31.45) as the internal standard. Two-dimensional spectra were obtained using standard pulse sequences from the Bruker software.

RESULTS

The cell wall of *S. melanosporofaciens* VKM Ac-1864^T contained 2.45% of teichoic acids phosphorus. The products of acid hydrolysis (2 N HCl, 100°C, 3 h) were glycerol mono- and bisphosphates, inorganic phosphate (electrophoresis in buffer A), glycerol, galactose (paper chromatography in system B), and glucosamine (paper chromatography in system C). Acid hydrolysates of the total ACP preparation isolated from the cell wall with 10% trichloroacetic acid comprised the same products as those from the cell wall. Electrophoresis of the total preparation revealed three fractions, which were accumulated by preparative electrophoresis in buffer A and studied separately.

Electrophoretic mobility relative to that of glycerol monophosphate (m_{GroP}) of fraction 1 was about 1.4. Glycerol, its mono- and bisphosphates, and mineral phosphate were identified after acid hydrolysis. Formation of the same products and of glycerol triphosphate as a result of alkaline hydrolysis indicated that fraction 1 was unsubstituted poly(glycerol phosphate) [11].

Fraction 2 had an electrophoretic mobility (m_{GroP}) of 0.82. Acid hydrolysates were found to contain glycerol mono- and bisphosphates, as well as mineral phosphorus, glycerol, and glucosamine.

The main products of HF hydrolysis were glycerol, mineral phosphate, and glycoside G1. The latter was detected in the neutral zone (electrophoresis in buffer A) and had a chromatographic mobility R_{Gro} of 0.7 in system B. It was stained by $AgNO_3$ but not by ninhydrin, contained equimolar amounts of glycerol and glucosamine, did not form formaldehyde in the course of periodate oxidation, and contained no reducing groups. The above characteristics were evidence of the pyranose form of glucosamine residues in the glycoside and of the presence of a bond between the amino sugar and glycerol via the glycoside hydroxyl of the amino sugar to C-2 of glycerol. This bond had an α configuration, as was evident from the data of NMR spectroscopic analysis [8]. Thus, glycoside G1 was determined as 2-acetamide-2-deoxy-α-D-glucosaminyl-(1⟶2)-glycerol.

The products of alkaline hydrolysis of fraction 2 included glycerol, glycerol mono- and bisphosphates, mineral phosphate, and phosphoric ester. The latter was not stained by ninhydrin; glycerol, glycerol mono- and bisphosphates, and glucosamine were detected after its acid hydrolysis. Apparently, the ester was a diglycerol phosphate with a phosphate group and N-acetylglucosamine residue at both C-2 of glycerol [11]. The above properties of the substance of fraction 2 are typical of 1,3-poly(glycerol phosphate) polymers partially substituted by N-acetylated α-glucosaminyl residues.

Fraction 3 had an electrophoretic mobility (m_{GroP}) of 0.3 and was stained gray by Isherwood's reagent. Acid hydrolysis revealed the presence of galactose and the products of degradation of teichoic acid from fraction 2 in trace amounts, which indicated that teichoic acid was not the main component of this fraction.

The non-fractionated total preparation of ACP from the *S. melanosporofaciens* cell wall was independently studied by NMR spectroscopy. The ^{13}C-NMR spectrum of the total preparation contained two series of signals (Table 1, Fig. f) of different integral intensity. The signals at δ 62.3, 66.2, and 77.3 corresponded to [–2)-*sn*-Gro-(3-P] residues unsubstituted in position C-1. The two signals at δ 67.57 and 70.97 belonged to [–1)-*sn*-Gro-(3-P] residues unsubstituted in position 2; other signals were identified as belonging to [–1)-*sn*-Gro-(3-P] residues substituted by α-N-acetylglucosamine at C-2. The anomer resonance region of the ^{13}C-NMR spectrum had two intensive signals from hexopyranoses (δ 98.32 and 104.8 in the ratio of 1 : 2) and a minor signal at δ 96.12 of the quaternary (the APT spectrum data) anomer carbon atom typical of the Kdn (3-deoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid) residue. The presence of Kdn was confirmed the by intensive signal at δ 39.9 typical of C-3 of deoxynonulosonic acid, which belonged to the $–CH_2$ group according to the APT spectrum data. In addition, an intensive signal from nitrogen-bearing carbon, at δ 54.99, and a signal typical of N-acetyl groups (CH_3CON, δ 23.4) were found. The analysis of the APT spectrum [12] made it possible to identify a nonprotonated anomer carbon atom (C-2) from nonulosonic acid (δ 96.12), the signals from $–CH_2O–$ groups at δ 61.89, 62.3, 65.9, 66.2, 66.6, 67.57, and 70.0, and two signals from CO groups: δ 174.57 and 175.8 (Table 1, Fig. 1).

The ^{1}H-NMR spectrum was decoded using the data from 2D homonuclear COSY and TOCSY spectra. The chemical shifts [13] and the analysis of constants of spin–spin interaction [14] of amino sugar residues pointed to the presence of α-D-N-acetylglucosamine. In addition, signals typical of axial and equatorial protons at C-3 of nonulosonic acid of the β-anomer, δ 1.84 (triplet) and 2.23 (doublet of doublets) were revealed. The spectrum additionally contained an intensive signal from the CH_3CON group at δ 2.06 and a minor sig-

Table 1. Chemical shifts of carbon atoms in the ^{13}C-NMR spectrum of anionic polymers from the cell wall of *S. melanosporofaciens* VKM Ac-1864^T

Residue	Chemical shifts, δ/ppm										
	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	CH_3	CO
→4)-β-Kdn-(2→	174.57	96.1	39.9	70.4	71.7	72.8	69.3	71.0	73.0		
9)											
↑											
β-D-Gal*p*-(1	104.8	72.25	74.0	70.0	76.4	62.3					
-1)-*sn*-Gro-(3-*P*-	66.6	76.4	65.9								
2)											
↑											
α-D-Glc*p*NAc-(1	98.32	54,99	72.26	71.40	73.06	61.89				23.4	175.8
-1)-*sn*-Gro-(3-*P*-	67.57	70.97	67.57								
-2)-*sn*-Gro-(3-*P*-	62.3	77.3	66.2								
sn-Gro-(3-*P*-	63.4	75.1	66.1								
2)											
|											
Lys	ND	53.3	27.8	22.1	32.8	40.4					

nal at δ 2.10, apparently belonging to the CH_3COO groups.

The proton and carbon spectra were decoded using two-dimensional spectroscopy COSY, TOCSY, ROESY, and HSQC. The analysis of the spectra showed the presence of two major polymers: 1,3-poly(glycerol phosphate), substituted for 57% by N-acetylated α-glucosaminyl residues, and a Kdn polymer (Table 1).

The conclusion about the structure of the latter polymer was made on the basis of the weak-field positions of C-4 and C-9 signals in the ^{13}C-NMR spectrum (Table 1) as compared with those of free β-Kdn [15] and the presence of correlation peaks H-1 β-Gal*p*/H-9 Kdn in the ROESY spectrum. The signals of Kdn terminal units were found neither in proton nor in carbon spectra, which indicates a chain length of no less than 20 repeating β-Kdn units bound by C-2 and C-4 Kdn and bearing β-Gal*p* by C-9, with *O*-acetyl groups bound to them in different positions. This is the first time a polymer of such structure has been found.

In addition to the above polymers, non-substituted 2,3- and 1,3-poly(glycerol phosphate) chains and terminal units of the following structure were revealed:

sn-Gro-(3-*P*
2)
|
Lys

This structure pertains to one of the above mentioned teichoic acids (Table 1; Fig. 1).

The finding of a Kdn polymer in *S. melanosporofaciens* VKM Ac-1864^T induced a more focused study of ACP in the strains of related species (*S. hygroscopicus* subsp. *hygroscopicus* VKM Ac-831^T, *S. endus* VKM Ac-1331^T, *S. endus* VKM Ac-129, *S. violaceusniger* VKM Ac-583^T, and *S. rutgersensis* subsp. *castelarensis* VKM Ac-832^T), in which only teichoic acids had been identified previously [5, 8]. The repeated investigation showed that the products of acid and alkaline hydrolyses of cell walls and total ACP preparations contained substances identical to those described previously, with the exception of additionally identified galactose (from a much greater amount of the cell wall preparation). The analysis of total ACP preparations by electrophoresis in buffer A revealed several polymers with electrophoretic mobility m_{GroP} 1.4, m_{GroP} 0.82–0.9 and m_{GroP} 0.3. The content of phosphorus of teichoic acids in the cell walls was 2.17 to 3.31%.

The analysis of the chemical and NMR spectroscopic characteristics of the ACP preparations from cell walls of *S. hygroscopicus* subsp. *hygroscopicus*, *S. violaceusniger*, *S. rutgersensis* subsp. *castelarensis*, and two *S. endus* strains confirmed the presence in each of them of the three previously described teichoic acids: predominant 1,3-poly(glycerol phosphate) partially substituted by α-glucosaminyl residues at glycerol C-2, some of which were N-acetylated (Table 2, Fig. 1); and minor amounts of 1,3- and 2,3-glycerolteichoic acids with no substitutes.

Hydroxamic reaction revealed *O*-acetyl residues in fraction 2 from all the strains. Acetic acid hydroxamate was identified chromatographically in system D through comparison with the standard sample and had chromatographic mobility R_f 0.54. The presence of galactose, which is not included in the structure of the identified teichoic acids, and a number of additional

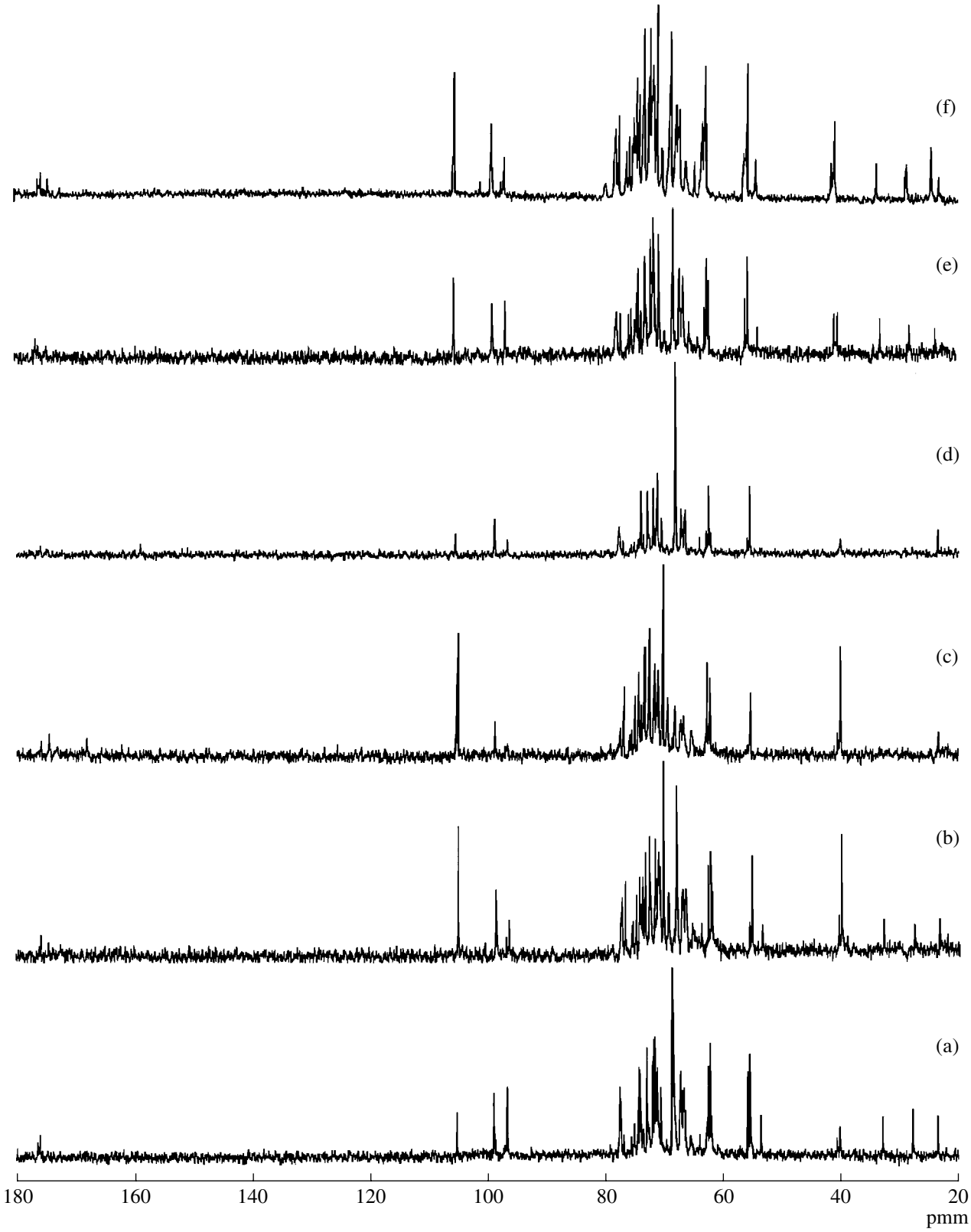

Fig. 1. The ^{13}C-NMR spectra of the total preparations of anionic carbohydrate-containing polymers from cell walls of streptomycetes under study. (a) *S. endus* VKM Ac-1331^{T}; (b) *S. endus* VKM Ac-129; (c) *S. hygroscopicus* subsp. *hygroscopicus* VKM Ac-831^{T}; (d) *S. rutgersensis* subsp. *castelarensis* VKM Ac-832^{T}; (e) *S. violaceusniger* VKM Ac-583^{T}; (f) *S. melanosporofaciens* VKM Ac-1864^{T}.

Table 2. Distinctive features of teichoic acids of strains under study (by results of the present work and previously published data [5, 8])

Characteristics	*S. melanosporofaciens* VKM Ac-1864^T	*S. rutgersensis* subsp. *castelarensis* VKM Ac-832^T	*S. hygroscopicus* subsp. *hygroscopicus* VKM Ac-831^T	*S. endus* VKM Ac-1331^T	*S. endus* VKM Ac-129	*S. violaceusniger* VKM Ac-583^T
1,3-poly(glycerol phosphate)						
Degree of substitution by α-N-acetylglucosamine and α-N-glucosamine, %	57	33	66	50	47	40
Share of α-N-acetylglucosamine, %	100	83	83	45	50	55
Cell wall or total ACP preparation: products of HF–hydrolysis:						
N-acetyl-glucosaminylglycerol (G1)	+	+	+	+	+	+
Glucosaminylglycerol (G2)[1]	–	+	+	+	+	+

Note: The cell walls of all the organisms studied in this work contain three teichoic acids each: predominant 1,3-poly(glycerophosphate) with α-glucosaminyl substitutes by glycerol C-2, some of which are N-acetylated; minor 1,3- and 2,3-poly(glycerophosphate) polymers containing no substitutes; and a Kdn polymer (of 3-deoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid) with β-galactose as a substitute at C-9 of nonulosonic acid. The products of cell wall acid hydrolysis and ACP acid hydrolysates (2 N HCl, 100°C, 3 h) comprise glycerol mono- and diphosphates, inorganic phosphate, glycerol, galactose, and glucosamine.

[1] Identification of glycoside G2 is described in detail in the works [5, 8].

specific signals in the NMR spectra (Table 1, Figure), led to the conclusion that the substance with electrophoretic mobility m_{GroP} 0.3 is a Kdn polymer identical to that found in *S. melanosporofaciens*.

DISCUSSION

The findings show that the cell walls of all the studied representatives of phylogenetically and phenotypically close organisms (*S. melanosporofaciens* VKM Ac-1864^T, *S. hygroscopicus* subsp. *hygroscopicus* VKM Ac-831^T, *S. violaceusniger* VKM Ac-583^T, *S. endus* VKM Ac-1331^T, *S. endus* VKM Ac-129, and *S. rutgersensis* subsp. *castelarensis* VKM Ac-832^T) contain three teichoic acids: predominant 1,3-poly(glycerol phosphate) with N-acetylated α-glucosaminyl substitutes by glycerol C-2, minor 1,3- and 2,3-poly(glycerol phosphate) polymers containing no substitutes, and previously unknown natural polymer Kdn (of 3-deoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid) with β-galactose as a substitute located at C-9.

Kdn oligomers and polymers with slightly different structures have been found previously in streptomycetes, potato scab pathogens [15, 16]. An acid polysaccharide with a Kdo-like sugar, a member of the family of the higher 3-deoxyulosonic acids that include Kdn, was found in the bacterium *Agrobacterium tumefaciens*, which causes root cancer in carrot [17]. Such structures localized on the cell surface of a phytopathogenic microorganism are supposed to play an important role in the infection process, determining the adsorption of a phytopathogen to a plant host cell [15–17]. The finding of a Kdn polymer in strains of "soil" species which had been traditionally considered as saprophytes (not included in the lists of phytopathogenic microorganisms [18, 19]) was quite unexpected. Elucidation of the question whether cell wall Kdn polymers/oligomers are indicators of phytopathogenicity of streptomycetes (actinomycetes) or, along with teichoic acids, are rather widespread among the organisms of this group is an object of further study.

The studied strains from different genera contained teichoic acids identical in core structure but differing in the number of α-glucosaminyl residues in the predominant teichoic acid (approximately 33 to 66%) and in the degree of their acetylation (45 to 100%) (Table 2). The above characteristics had a fairly high degree of reproducibility (cultures in the logarithmic growth phase) in different variants of the experiments (the data from the present and previously published works [5, 8]). These characters may be considered as specific to the above organisms and may be useful for the identification of this group of streptomycetes in combination with other phenotypic and genotypic characteristics. In particular, the representatives of the species *S. hygroscopicus* subsp. *hygroscopicus* and *S. endus* with a high level of DNA–DNA similarity (92%) can be well-distinguished by the degree of acetylation of α-glucosaminyl substitutes [7]. It can also be noted that *S. melanosporofaciens* is characterized by the presence of a single glycoside, N-acetyl glucosaminylglycerol, in cell wall HF hydrolysates and in the total polymer fraction (due to complete N-acetylation of α-glucosaminyl sub-

stitutes) revealed by descending paper chromatography in system B. All other organisms have one additional glycoside: glucosaminylglycerol (Table 2).

The question of whether the quantitative characteristics reflecting the nature of α-glucosaminyl substitutes of teichoic acids correlate with the species affiliation of phylogenetically close organisms of the group under study and whether the above quantitative characteristics may be species markers needs further (including taxonomic) investigation with extended sampling of the relevant strains.

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