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Cell Wall Teichoic Acids of Actinomycetes of Three Genera of the Order *Actinomycetales*

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Abstract—The structures of cell wall teichoic acids of the members of newly recognized genera of the order *Actinomycetales* were studied. *Planotetraspora mira* VKM Ac-2000^T contains two types of teichoic acids: 2,3-poly(glycerol phosphate) substituted with α-D-Gal*p* at C-1 of glycerol and 1,3-poly(glycerol phosphate) substituted with α-L-Rha*p* at OH-2 of glycerol (60%). *Herbidospora cretacea* VKM Ac-1997^T contains the chains of 1,3-poly(glycerol phosphate) partially substituted with α-D-Gal*p* and α-D-Gal*p*NAc at C-2 of glycerol. The majority of α-D-galactopyranosyl residues are substituted at OH-3 with a sulfate. The aforementioned teichoic acids have not been found in bacteria thus far. *Actinocorallia herbida* VKM Ac-1994^T contains poly(galactosylglycerol phosphate), with the β-Gal*p*-(1→2)-Gro-*P* repeating units being linked via the phosphodiester bonds between the OH-3 of glycerol and OH-6 of galactose. Earlier, this structure was found in the cell wall of *Actinomadura madura*. The polymer structures were determined by chemical analysis and using ^{13}C-NMR spectroscopy. The results show that teichoic acids are widespread in the order *Actinomycetales*.

Key words: *Planotetraspora*, *Herbidospora*, *Actinocorallia*, cell wall, poly(glycerol phosphate), poly(glycosylglycerol phosphate), sulfate, NMR spectroscopy

During recent decades the structures of teichoic acids of actinomycetes have been intensely studied so as to put in order the taxonomy of this large group of soil microorganisms, many of which are used in medicine and national economy. Our previous studies of the cell walls of the species belonging to the genera *Nocardiopsis* [1], *Streptomyces* [2, 3], and *Glycomyces* [4] showed that the structures of anionic polymers may serve as chemotaxonomic markers for determination of the actinomycete species.

The order *Actinomycetales* includes approximately 30 families, each including several genera [5]. However, the extent to which teichoic acids are spread in this order is studied insufficiently. Teichoic acids are studied in the genera *Streptomyces*, *Glycomyces*, *Actinomadura*, *Nocardioides*, *Nocardiopsis*, and some others [1].

Teichoic acids of actinomycetes are characterized by a great variety of structures. Analysis of new genera of these microorganisms is interesting with respect to studying the distribution of these polymers and finding new structures of bacterial cell surface as well.

The purpose of this work was to broaden the search for anionic polymers of the cell walls of the bacteria belonging to the order *Actinomycetales*, to detect teichoic acids, and (if the latter are contained in the cell wall) to determine their structures in the typical species of the genera *Planotetraspora*, *Herbidospora*, and *Actinocorallia*.

MATERIALS AND METHODS

Strains. In this study, we used actinomycetes *Planotetraspora mira* VKM Ac-2000^T, *Herbidospora cretaceae* VKM Ac-1997^T, and *Actinocorallia herbida* VKM Ac-1994^T obtained from the All-Russian Collection of Microorganisms (VKM).

Culturing conditions. The cultures were grown in flasks containing peptone–yeast medium (100 ml) [6] on a shaker at 28°C. One-day-old cultures obtained under

Abbreviations: APT) attached-proton test; COSY) two-dimensional ^{1}H,^{1}H correlation spectroscopy; HMQC) H-detected heteronuclear multiple-quantum coherence spectroscopy; ROESY) nuclear Overhauser effect spectroscopy; HMBC) heteronuclear ^{1}H,^{13}C multiple-bond correlation; HSQC) heteronuclear single-quantum coherence spectroscopy; SSCC) spin-spin coupling constant.

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the same conditions served as inoculates. The biomass for cell wall isolation was harvested at the logarithmic growth phase (17-24 h), washed with distilled water, frozen, and stored at −20°C.

Cell walls were obtained by disrupting the mycelium suspended in 1% aqueous SDS by sonication using an ultrasound disintegrator as described earlier [7]. The cell wall preparations were lyophilized.

Isolation of polymers. The polymers were obtained from lyophilized cell walls by two consecutive 24-h extractions with 10% trichloroacetic acid at 2-4°C. The extracts were separated from the cell wall debris, pooled, dialyzed against distilled water, and lyophilized.

Acid hydrolysis. Phosphate esters, polyols, amino sugars, and monosaccharides were identified after hydrolysis of the cell wall, polymers, and glycosides with 2 M HCl at 100°C for 3 h. The glycosides were obtained by hydrolyzing the samples with 48% HF at 4°C for 24 h. The hydrolyzate was treated with Dowex 2×4 (Dow, USA) (the CO_3^{2-} form), lyophilized, and chromatographed.

Alkaline hydrolysis. The polymers were hydrolyzed with 1 M NaOH at 100°C for 3 h. The hydrolyzate was treated with Dowex 8×50 (Dow) (the NH_4^+ form) and lyophilized.

Enzymatic hydrolysis with phosphomonoesterase (EC 3.1.31; Sigma, USA) was performed in ammonium acetate buffer (pH 9.8) at 37°C for 18 h.

O-Acetyl groups were detected by the reaction with hydroxylamine as described earlier [8].

Electrophoresis and chromatography were performed on Filtrak FN-13 paper (Germany). Phosphate esters and amino sugars were separated by electrophoresis in pyridine-acetate buffer, pH 5.6, at 20 V/cm for 3-4 h. Monosaccharides, glycosides, amino sugars, and polyols were separated by descending chromatography in pyridine–benzene–butan-1-ol–water (3 : 1 : 5 : 3 v/v).

Reagents used for the detection of compounds were as follows: the Isherwood reagent for phosphate esters [9], 5% aqueous $AgNO_3$ for polyols, ninhydrin for amino sugars, aniline phthalate for monosaccharides, and 2% $FeCl_3$ in 0.1 M HCl for acetyl hydroxamate. Phosphorus was assayed as described in [10]; amino sugars, in [11]; polyols and formaldehyde, in [12]; galactose was determined with anthrone.

Quantitative determination of sulfate and galactose. The preparation (19.67 mg) was hydrolyzed by heating in 1 ml of 2 M trifluoroacetic acid supplemented with myoinositol (0.9 mg/ml) at 100°C for 8 h. Sulfate contained in the hydrolyzate aliquots (0.2 ml) was quantitatively determined using a turbidimetric method [13]. The remaining hydrolyzate was treated with $NaBH_4$ and acetylated under standard conditions [14]. Gas-liquid chromatography was conducted using a Hewlett-Packard 5890A chromatograph equipped with an HP 3393A flame ionization detector and an HP Ultra-2 capillary column (Hewlett-Packard, USA). Chromatography was performed in a nitrogen flow at temperatures programmed from 200 to 290°C at the rate of 10°C/min. Quantitative determination of galactose was performed by comparing the peak corresponding to dulcitol peracetate with the peak corresponding to the internal standard (myoinositol peracetate).

Infrared spectra were recorded on a Specord M-80 spectrometer (Carl-Zeiss, Germany) in a KBr tablet.

NMR spectra were acquired from 2-3% solutions of teichoic acids in D_2O at 30°C on a DRX-500 device (Bruker, Germany) using acetone as a standard (2.225 and 31.45 ppm in ^1H- and ^{13}C-spectra, respectively). Two-dimensional spectra were obtained using a Bruker software package.

RESULTS AND DISCUSSION

Cell wall teichoic acids of *Planotetraspora mira* VKM Ac-2000^T. The genus *Planotetraspora* discovered in 1993 was classified with the family Streptosporangiaceae based on the analysis of the 16S rDNA [15]. The cell wall of a typical strain of this genus, *P. mira* VKM Ac-2000^T, studied in this work contained 2.5% organic phosphorus. Acid hydrolysis of the preparation of this cell wall produced glycerol mono- and diphosphates, glycerol, galactose, rhamnose, and inorganic phosphate, which is suggestive of the presence of glycerol teichoic acid in this preparation. The organic phosphorus-containing preparation was isolated from the cell wall and studied by chemical methods and NMR spectroscopy.

Alkaline hydrolysis of the preparation yielded several phosphate esters, with glycerol mono- and diphosphates and the E1 and E2 esters being predominant. Glycerol mono- and diphosphates were identified using electrophoresis in pyridine-acetate buffer and compared to the reference samples. The E1 and E2 esters were accumulated using preparative electrophoresis and studied.

Acid hydrolysis of the E1 ester (m_{GroP} 0.43) yielded galactose and glycerol monophosphate in equimolar amounts; phosphomonoesterase-catalyzed hydrolysis, glycoside 1 and inorganic phosphate. Glycoside 1 contained equimolar amounts of galactose and glycerol. Periodate oxidation of glycoside 1 resulted in the production of formaldehyde and glycerol in 1 : 1 ratio. Given the presence of the pyranose form of galactose (see NMR spectroscopic data), this finding suggests that the glycerol residue is substituted with galactose at the primary hydroxyl.

Thus, these data indicate that E1 is the monophosphate of galactosyl-(1→1)-glycerol.

Upon acid hydrolysis, the E2 ester (m_{GroP} 0.73) gave rhamnose, glycerol mono- and diphosphates, and glycerol; phosphomonoesterase cleaved 50% of its phosphorus. The Gro/Rha/P molar ratio in this ester was 2 : 1 : 2.

The hydrolysis of the preparation with HF, besides glycoside 1, also produced glycoside 2 (with the Gro/Rha molar ratio of 1 : 1), with no formaldehyde being formed

upon its periodate oxidation. This finding is indicative of the substitution of the secondary hydroxyl in glycerol with rhamnose. Therefore, the E2 ester apparently has the following structure: Rha-(1→2)-Gro-*P*-Gro-2-*P*.

The presence of glycerol phosphodiester among the products of alkaline hydrolysis is suggestive of the 1,3-poly(glycerol phosphate) chain. The production of this ester may be accounted for based on the mechanism of alkaline hydrolysis of the 1,3-poly(glycerol phosphate) chain in part substituted with a glycosyl at the C-2 of glycerol [16].

On the other hand, the E1 ester could not originate from the structure described above. It is known that the phosphomonoester of glycosyl-(1→1)-glycerol is produced upon hydrolysis of glycerol teichoic acids of two possible structures: glycosylated 2,3-poly(glycerol phosphate) or poly(glycosylglycerol phosphate) chains [17].

These data suggest that the cell wall may contain two teichoic acids with differing structures. However, we could not separate the polymers by ion exchange and gel chromatography.

The question on the presence of two polymers in the preparation analyzed was answered using NMR spectroscopy.

The ^{13}C-NMR spectrum of the preparation contained two signals in the region of anomeric carbon atoms (99.3 and 100.3 ppm) and one C-*C*H$_3$ signal (18.0 ppm) (Fig. 1). The other signals were detected within the 61-80 ppm region, which is typical for the carbon atoms bonded to one oxygen atom (Table 1). Two major signals were also detected in the region characteristic of anomeric protons in the ^{1}H-NMR spectrum (4.975 and 5.20 ppm) (Fig. 2, Table 1), and the C-C*H*$_3$ signal was at 1.31 ppm. The ^{31}P-NMR spectrum contained a very broadened signal at 3.5 ppm.

The ^{1}H- and ^{13}C-NMR spectra were assigned using two-dimensional homonuclear ^{1}H/^{1}H-COSY, TOCSY, and ROESY spectroscopy and heteronuclear ^{1}H/^{13}C-HSQC and HMBC-TOCSY spectra.

Analysis of the two-dimensional spectra revealed two different polymer types in the preparation studied.

One of them was 2,3-poly(glycerol phosphate) containing α-galactopyranose at the C-1 of glycerol. A low-field position of the C-1 signal of glycerol (68.1 ppm) in the ^{13}C-NMR spectra and the presence of a correlation peak H-1 (Gal)/H′-1 (Gro) confirmed the substitution type.

The second polymer was 1,3-poly(glycerol phosphate) substituted at C-2 by 60% with α-rhamnopyranosyl residues. The substitution at C-2 of glycerol was confirmed by the presence of a correlation peak H-1 (Rha*p*)/H-2 (Gro) in the ROESY spectrum and a low-filed position of the C-2 (Gro) signal in the ^{13}C-NMR spectrum (78.5 ppm).

The glycosides contained in the HF-hydrolyzate were isolated by paper chromatography, and their structures were determined using one-dimensional ^{1}H- and ^{13}C-NMR spectra and two-dimensional COSY, ROESY, and HSQC spectra (Table 1).

Thus, the cell wall of *Planotetraspora mira* contains two teichoic acids: 2,3-poly(glycerol phosphate) substituted with α-galactopyranose and 1,3-poly(glycerol phosphate) substituted by 60% with α-rhamnopyranose.

Earlier, 2,3-poly(glycerol phosphates) substituted with α-D-glucose [4], β-D-glucose [18], and a disaccharide comprised of N-acetylgalactosamine and galactose [19], as well as unsubstituted chains [2, 20] have been identified.

Rhamnose was previously found as monomeric glycosyl substituent of the poly(ribitol phosphate) teichoic acids [21, 22], in the tetrasaccharide substituent of poly(ribitol phosphate) [23] and in the trisaccharide substituent of poly(arabitol phosphate) [24].

2,3-Poly(glycerol phosphate) substituted with galactosyl residues and 1,3-poly(glycerol phosphate) substituted with rhamnosyl residues have not been previously found in the bacterial cell walls.

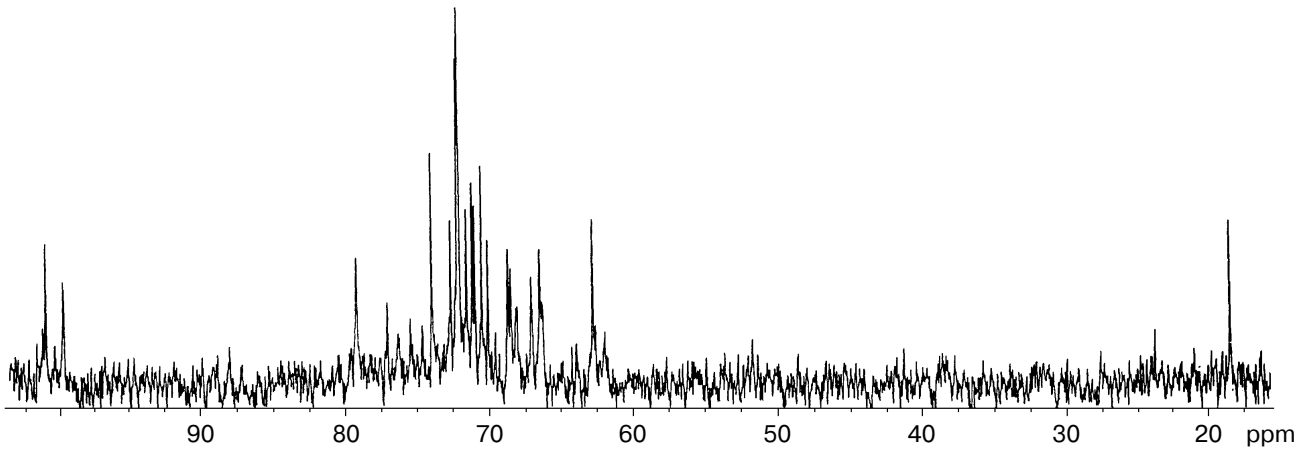

Fig. 1. ^{13}C-NMR spectrum of the preparation of cell wall teichoic acids of *Planotetraspora mira* VKM Ac-2000^{T}.

Table 1. NMR-spectroscopic data on the cell wall teichoic acids of *Planotetraspora mira* VKM Ac-2000^T

Residue	Chemical shift (δ), ppm			
-2)-*sn*-Gro-(3-*P*-	H-1	4.13	C-1	68.1
1	H-1'	4.02	C-2	76.5
	H-2	4.13	C-3	66.55
	H-3,3'	4.08		
↑				
α-Gal*p*-1	H-1	5.20	C-1	99.3
	H-2	3.84	C-2	69.9
	H-3	3.915	C-3	70.65
	H-4	4.035	C-4	70.5
	H-5	4.15	C-5	72.2
	H-6,6'	3.77	C-6	62.4
sn-Gro	H-1	4.13	C-1	70.6
1	H-1'	4.02	C-2	70.7
	H-2	4.13	C-3	64.15
	H-3,3'	4.08		
↑				
α-Gal*p*-1	H-1'	5.20	C-1	99.4
	H-2	3.84	C-2	69.8
	H-3	3.915	C-3	71.5
	H-4	4.035	C-4	70.6
	H-5	4.15	C-5	72.4
	H-6,6'	3.77	C-6	62.4
-1)-*sn*-Gro-(3-*P*-	H-1,1'	4.01	C-1	67.8
	H-2	4.08	C-2	70.7
	H-3,3'	3.96	C-3	67.8
-1)-*sn*-Gro-(3-*P*-	H-1	4.17	C-1	66.04
2	H-1'	4.11	C-2	78.7
	H-2	4.09	C-3	66.0
	H-3	4.11		
	H-3'	4.06		
↑				
α-Rha*p*-1	H-1	4.975	C-1	100.6
	H-2	4.01	C-2	71.9
	H-3	3.845	C-3	71.7
	H-4	3.45	C-4	73.7
	H-5	3.96	C-5	70.3
	H-6	1.31	C-6	18.3
sn-Gro	H-1	4.17	C-1	61.8
2	H-1'	4.11	C-2	80.1
	H-2	4.09	C-3	62.7
	H-3	4.11		
	H-3'	4.06		
↑				
α-Rha*p*-1	H-1	4.975	C-1	100.5
	H-2	4.01	C-2	71.8
	H-3	3.845	C-3	71.5
	H-4	3.45	C-4	73.4
	H-5	3.96	C-5	70.2
	H-6	1.31	C-6	18.0

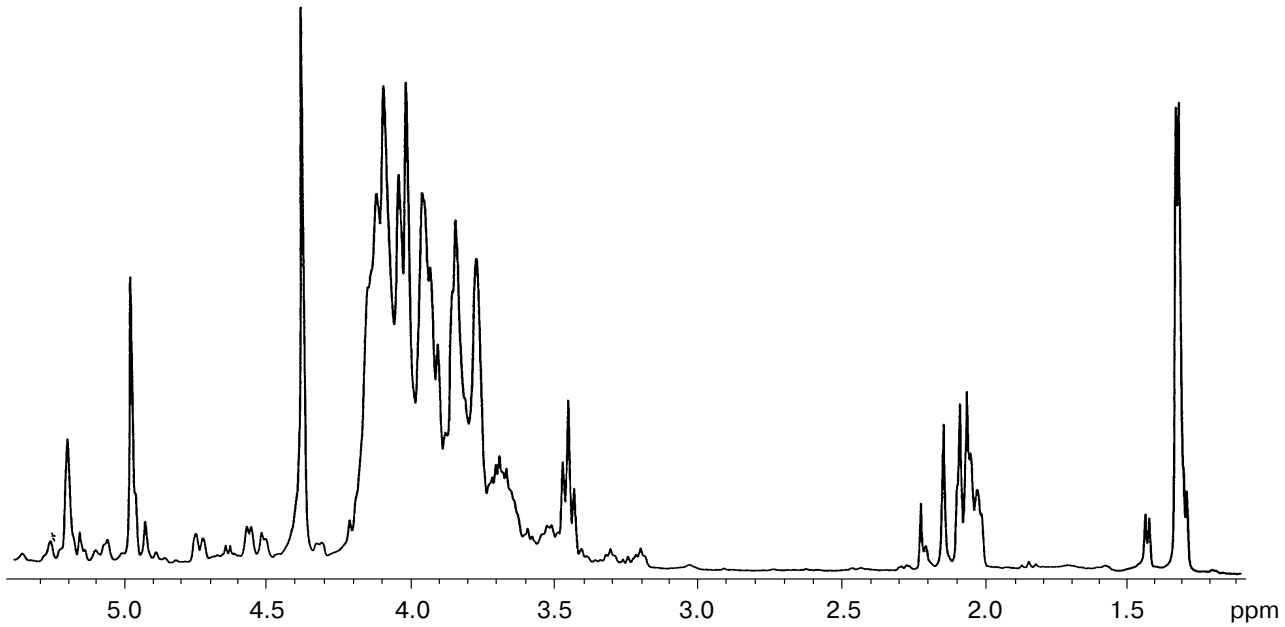

Fig. 2. ^{1}H-NMR spectrum of the preparation of cell wall teichoic acids of *Planotetraspora mira* VKM Ac-2000^{T}.

Cell wall teichoic acid of *Herbidospora cretaceae* VKM Ac-1997^{T}. *Herbidospora cretaceae* VKM Ac-1997^{T} is a typical species of the new genus *Herbidospora* (the family Streptosporangiaceae) that includes actinomycetes isolated from soil and plants. The genus was distinguished based on the aggregate morphological and chemotaxonomic characteristics and phylogenetic analysis [25].

In this study, the polymer was isolated from the cell walls containing 2% organic phosphorus. The products of acid hydrolysis of this polymer included glycerol mono- and diphosphates, galactose, galactosamine, glycerol, and inorganic phosphate. The major esters produced upon its alkaline hydrolysis were glycerol mono- and diphosphates. With regard for the aforementioned reasons, the presence of a small amount of glycerol phosphodiesters (of which one contained galactose and the other contained galactosamine) provides evidence in favor of 1,3-poly(glycerol phosphate) chain(s) in part substituted with the glycosyl substituents. The small amount of sugar-containing glycerol diphosphates did not allow us to study their structure. A solution to this problem was to analyze these polymers by NMR spectroscopy.

The ^{13}C-NMR spectrum contained two intensive signals at 67.4 and 70.65 ppm, which are typical for unsubstituted 1,3-poly(glycerol phosphate) chain, and minor signals, two of which were in the resonance region of anomeric carbon atoms (99.1 and 98.2 ppm; Table 2).

In the ^{1}H-NMR spectrum, signals of lower intensity were also observed along with the signals produced by free poly(glycerol phosphate) chain. Two signals of lower intensities were recorded in the resonance region of pyranoses with α-configuration of the glycoside site. In a more high-field region, two signals with spin–spin coupling constants (SSCCs) typical for H-3 (4.57 ppm) and H-4 (4.38 ppm) of pyranose in galactose configuration were detected (Table 2). Chemical shift of the H-3 signal was observed at unusually low-field position compared to that characteristic of free galactopyranose [26].

The ^{1}H-NMR spectrum was interpreted using two-dimensional COSY and TOCSY spectroscopy; the ^{13}C-NMR spectrum, two-dimensional heteronuclear HSQC spectroscopy (Fig. 3). The analysis of the spectra showed that the most intensive signals were produced by the 1,3-poly(glycerol phosphate) chain. The signals with lower intensities belonged to the glycerol phosphate units substituted at C-2 and two sugar residues (unsubstituted α-Gal*p*NAc and α-Gal*p* substituted at the C-3 with an electronegative substituent (78.9 ppm)). The low-filed position of the signal is characteristic of galactose glycosylated at C-3. However, such a low-field position of the H-3 signal in the proton spectrum is not characteristic of glycosylation. The overall low-field shift of H-3 and C-3 signals may be due only to the presence of an electronegative group at C-3 (sulfate, nitrate, etc.).

To determine the substituent nature, we recorded an infrared spectrum of the preparation. The infrared spectrum contained an intensive absorbance band at 1232 cm^{-1} characteristic of all sulfates and an additional absorbance band at 835 cm^{-1} that can be assigned to the secondary equatorial sulfate (e.g., at C-3 of galactopyranose residue [27]). Quantitative determination of sulfate by turbidimetric method after complete acid hydrolysis of the preparation and simultaneous determination of galactose in the same hydrolyzate by gas–liquid chromatography in the form of dulcitol peracetate (1.85 and 3.8%,

Table 2. NMR-spectroscopic data on the cell wall teichoic acids of *Herbidospora cretaceae* VKM Ac-1997^T

Residue	Chemical shift (δ), ppm			
-1)-*sn*-Gro-(3-*P*-	C-1	67.4	H-1,1'	3.98
	C-2	70.65	H-2	4.07
	C-3	67.4	H-3,3'	3.92
-1)-*sn*-Gro- (3-*P*-	C-1	66.4	H-1,1'	4.05
2	C-2	76.6	H-2	4.07
↑	C-3	65.5	H-3,3'	4.04
α-D-Gal*p*-1	C-1	99.1	H-1	5.28
3	C-2	68.1	H-2	3.98
|	C-3	78.9	H-3	4.57
SO_3^-	C-4	68.8	H-4	4.38
	C-5	72.0	H-5	4.20
	C-6	62.2	H-6,6'	3.77
-1)-*sn*-Gro- (3-*P*-	C-1	66.4	H-1,1'	4.05
2	C-2	76.6	H-2	4.07
	C-3	65.5	H-3,3'	4.04
↑				
α-D-Gal*p*NAc-1	C-1	98.2	H-1	5.12
	C-2	51.0	H-2	4.22
	C-3	68.85	H-3	3.98
	C-4	69.7	H-4	4.03
	C-5	72.35	H-5	4.21
	C-6	62.45	H-6,6'	3.77
	CH3	23.4		
	CO	175.3		

respectively) showed that the sulfate/galactose molar ratio in the initial polymer is 3 : 4.

In addition, the hydroxamate reaction showed that the cell wall of the actinomycete studied contains O-acetyl groups.

Thus, the cell wall of *Herbidospora cretaceae* contains 1,3-poly(glycerol phosphate) chains with α-N-acetylgalactosaminyl and α-galactosyl substituents, the majority of the latter having a sulfate at C-3, which increases the negative charge on the cell surface.

The presence of sulfate in the teichoic acid has not been reported before. Sulfuric acid residues were found in the cell wall polysaccharide of *Arthrobacter* sp. AT-25 [28] and in the extracellular polysaccharides of Gram-positive and Gram-negative bacteria [29, 30].

Teichoic acid of *Actinocorallia herbida* VKM Ac-1994^T. The genus *Actinocorallia* of the family Thermomonosporaceae was described in 1994 [31]. The cell wall of a typical species of this genus, *A. herbida* VKM Ac-1994^T, studied in this work contains approximately 2% organic phosphorus. Acid hydrolysis of the cell wall yielded glycerol monophosphate, galactose, glycerol, and inorganic phosphate; the hydroxamate reaction revealed the presence of O-acetyl groups. The preparation isolated from the cell wall gave the same products upon acid hydrolysis, but was stable when heated with alkali. The molar ratio of the polymer components Gro/*P*/Gal was 1 : 1.04 : 0.9.

The hydrolysis of the teichoic acid with HF produced a glycoside which was accumulated and studied. Acid hydrolysis of this glycoside yielded equimolar amounts of glycerol and galactose. Periodate oxidation of the glycoside did not result in formaldehyde production, which indicates that galactose (in pyranose configuration) is linked to glycerol at C-2.

The stability of the polymer under alkaline conditions and the absence of glycerol diphosphate among the products of acid hydrolysis are suggestive of the poly(glycosylglycerol phosphate) nature of the teichoic acid, in which the glycoside bond is located at C-2 rather than C-1 of glycerol. A polymer is not hydrolyzed with alkali when the phosphodiester bond is formed between C-6 of the glycosyl residue and C-3 of glycerol (i.e., when the polymer lacks the adjacent hydroxyl groups, which hampers cyclic phosphate formation and prevents the chain from breaking) [16].

When studying the ^{13}C-NMR spectrum of the polymer, we found that the teichoic acid is a poly(β-galacto-

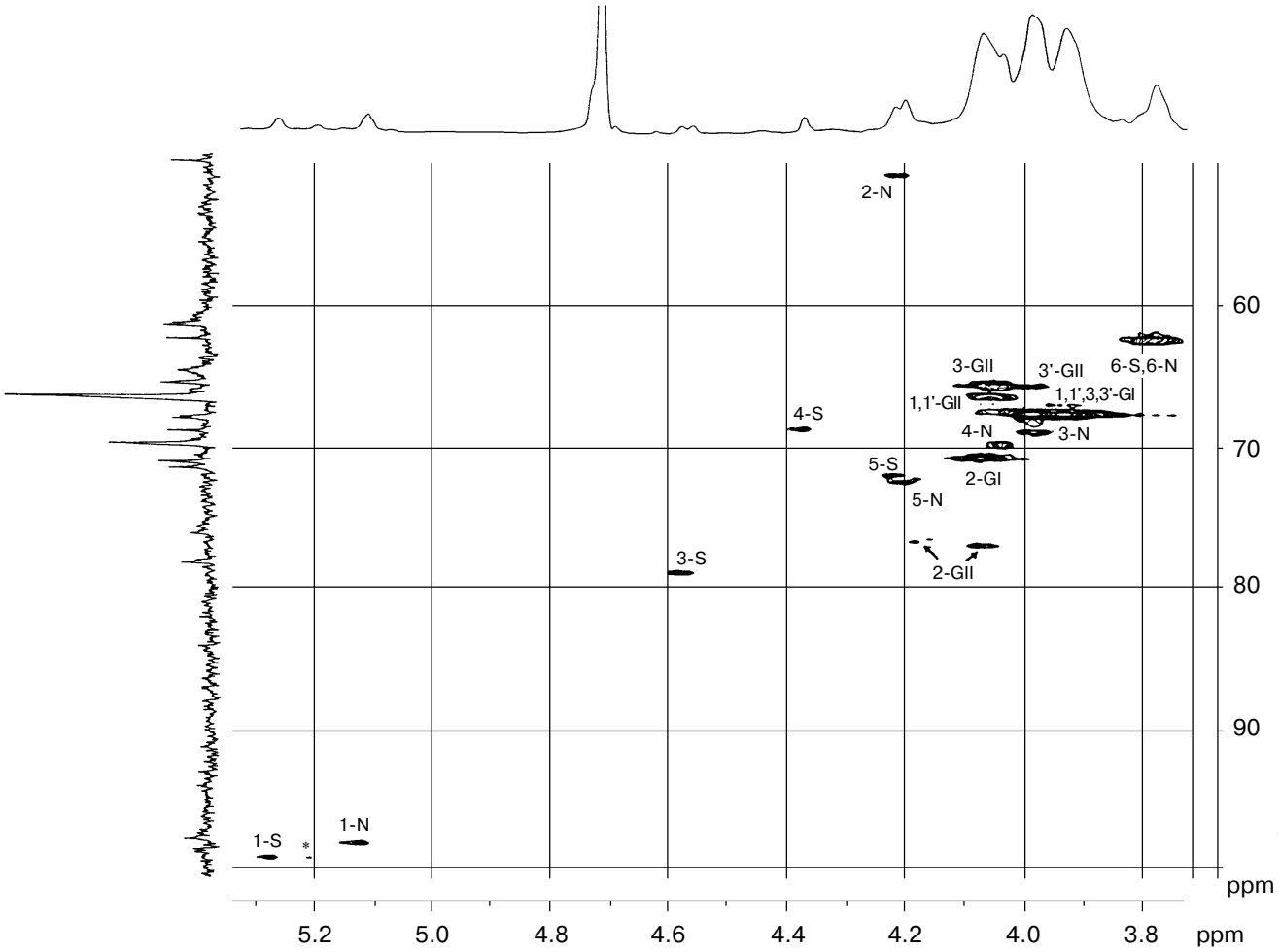

Fig. 3. The HSQC-spectrum of 1,3-poly(glycerol phosphate) chains from the cell wall of *Herbidospora cretaceae* VKM Ac-1997^T. Designations: Gal*p*NAc, N; Gal*p*SO_3, S; unsubstituted Gro*P*, GI; Gro*P* substituted at C-2, GII; *, correlation peak of C1/H1 sulfate-free galactose residues (minor).

syl-1→2-glycerol phosphate) in which the phosphodiester bond links C-6 of galactose and C-3 of glycerol (Table 3). The spectrum of the polymer studied is completely identical to the spectrum of one teichoic acid of the cell wall of *Actinomadura madura* INA 00018, which contains similar poly(galactosylglycerol phosphate) and poly(3-O-methylgalactosylglycerol phosphate) chains [32].

Thus, the results of this work demonstrate that broadening the range of actinomycetes studied allows

Table 3. ^{13}C-NMR chemical shifts for the cell wall teichoic acids of *Actinocorallia herbida* VKM Ac-1994^T

Residue	Chemical shifts (δ), ppm					
	C-1	C-2	C-3	C-4	C-5	C-6
2-*sn*-Gro- (3-*P*-	66.4	76.6	65.5			
↑						
-6-β-D-Gal*p*-1	99.1	68.1	78.9	68.8	72.0	62.2

describing new, previously unknown structures of cell wall polymers. This is interesting *per se* because it broadens our notion on the bacterial cell surface.

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