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Carbohydrate-Containing Polymers of the Cell Wall of the Thermophilic Streptomycete *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* VKM Ac-1857^T

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Abstract—Anionic polymers of the cell surface of a thermophilic streptomycete were investigated. The cell wall of *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* VKM Ac-1857^T was found to contain polymers with different structure: teichoic acid – 1,3-poly(glycerol phosphate), disaccharide-1-phosphate polymer with repeating unit -6)-α-Gal*p*-(1→6)-α-Glc*p*NAc-P-, and polysaccharide without phosphate with repeating unit →6)-α-Gal*p*NAc-(1→3)-β-Gal*p*NAc-(1→. Disaccharide-1-phosphate and polysaccharide without phosphate have not been described earlier in prokaryotic cell walls.

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Cell walls of studied microorganisms belonging to the *Actinomycetales* order usually contain various anionic polymers: in particular, teichoic and teichuronic acids and sugar-1-phosphate polymers found in some genera of this order; carbohydrate-containing polymers without phosphate are rarely found [1-4]. Negative charge is the common feature of anionic polymers. Moreover, simultaneous presence of several polymers with various negative charge density is typical of cell walls of actinomycetes [1, 5, 6].

The goal of the present work was to study the anionic polymers of cell wall of a typical strain of thermophilic alkali-stable streptomycete belonging to a special group of the *Streptomyces* genus [7]. These microorganisms are able to grow at elevated temperature (25-55°C) and numerically and phenetically differ from mesophilic streptomycetes [7]. To reveal a wide variety of natural anionic polymers, structural studies of cell wall polymers are necessary; molecular studies are also especially important for determination of internal taxonomy of a genus.

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MATERIALS AND METHODS

For this study, we took the typical thermophilic streptomycete strain *Streptomyces thermoviolaceus* subsp. *thermoviolaceus* VKM Ac-1857^T from the All-Russian Collection of Microorganisms (VKM). Cells were grown in shaker flasks containing 100 ml of peptone-yeast medium (pH 7.2-7.4), 28°C [8]. For isolation of cell wall, biomass was harvested in the logarithmic growth phase (17-20 h), washed with distilled water, and stored at –20°C. Cell wall preparation was obtained after ultrasonication of cell suspension in 0.5% SDS, subsequent centrifugation, and lyophilization [9].

Anionic polymers were isolated from cell wall by fractional extraction with 10% trichloroacetic acid (TCA) (2-3 times for 24 h, 4°C); individual polymers were obtained by preparative electrophoresis and gel chromatography. Chromatography and electrophoresis were preformed using FN-13 paper from Filtrak (Germany). Phosphoric ester, lysine, its derivatives, and polymers were separated by electrophoresis in pyridine–acetate

buffer 1 (pH 5.5-5.6, 20 V/cm, 3-4 h). For descending paper chromatography (PC), the following solvent systems were used: to separate monosaccharides and polyols—*n*-butan-1-ol–pyridine–benzene–H_2O (5 : 3 : 1 : 3 v/v); to separate aminosaccharides, lysine, and its derivatives—pyridine–ethyl acetate–acetic acid–H_2O (5 : 5 : 1 : 3 v/v).

Teichoic acids and phosphoric esters were detected with the Ischerwood reagent; monosaccharides with aniline phthalate; polyols and monosaccharides with 5% $AgNO_3$ ammonium solution; aminosaccharides, lysine, and its amide with ninhydrin; lysine hydroxamate with $FeCl_3$ [10]. After autohydrolysis on long incubation, the total polymer preparation was separated on a column (75 × 1.8 cm) with TSK 40 S gel from Toyo Soda (Japan), elution with 1% AcOH being monitored using a differential refractometer from Knauer (Germany).

Ammonolysis and hydroxylaminolysis of cell walls were performed as described earlier [10]; lysine amide and its hydroxamate were detected by PC and electrophoresis and comparison with the standards.

Preliminary chemical analysis of the primary structure of polymers was based on molecule degradation by various agents (heating with acids and alkali and enzymatic hydrolysis), investigation of hydrolyzate products, and subsequent reconstruction of the initial structure of the studied polymer.

For identification of phosphoric esters, polyols, monosaccharides, aminosaccharides, and lysine, acidic hydrolysis of polymers was performed in 2 M HCl for 3 h at 100°C. Disaccharide-1-phosphate polymer was hydrolyzed in 0.1 M HCl for 10 min and for 1 h at 100°C.

Alkaline hydrolysis was performed in 1 M NaOH for 3 h at 100°C, and the hydrolyzate was treated by Dowex 8X50 from Dow (USA) (NH_4^+-form) and lyophilized [10].

The NMR spectra were recorded for 2-3% solutions in D_2O at 30°C using a DRX-500 spectrometer from Bruker (Germany). Chemical shifts were measured with respect to the following standards: ^1H – internal TSP (sodium salt of 3-(trimethylsilyl)-3,3,2,2-tetradeuteropropionic acid), δ_H 0.0; ^{13}C – internal acetone, δ_C 31.45; ^{31}P – external (in capillary) 80% phosphoric acid, δ_P 0.0. The 2D NMR spectra were recorded according to the standard Bruker procedures. The HMBC spectra were optimized for spin-coupling constant $J_{H,C}$ 5 Hz; mixing time for ROESY was 100 msec. The ^1H,^{13}C HMQC spectra were optimized for spin-coupling constant $^3J_{H,C}$ 8 Hz.

RESULTS AND DISCUSSION

The lyophilized cell wall of *S. thermoviolaceus* subsp. *thermoviolaceus* VKM Ac-1857^T was treated with TCA, and the extracts were dialyzed against distilled water and lyophilized. Acidic degradation of preparations (2 M HCl, 3 h, 100°C) yielded galactose, glucosamine, galactosamine, lysine, glycerol, and its mono- and diphosphates. Electrophoresis in acetate-pyridine buffer 1 showed that the preparations are heterogeneous and most likely contain several organophosphorus compounds (staining with the Ischerwood reagent) moving to the anode with mobility m_{GroP} = 0.79 and 0.47 and also a neutral compound (staining with $AgNO_3$).

Ammonolysis and hydroxylaminolysis of *S. thermoviolaceus* cell walls yielded lysine, its amide, and hydroxamate; these compounds were identified by PC and electrophoresis and comparison with the standards. A set of phosphoric esters, neutral sugars, and aminosaccharides in all preparations was identical; in further studies a total pooled preparation was used.

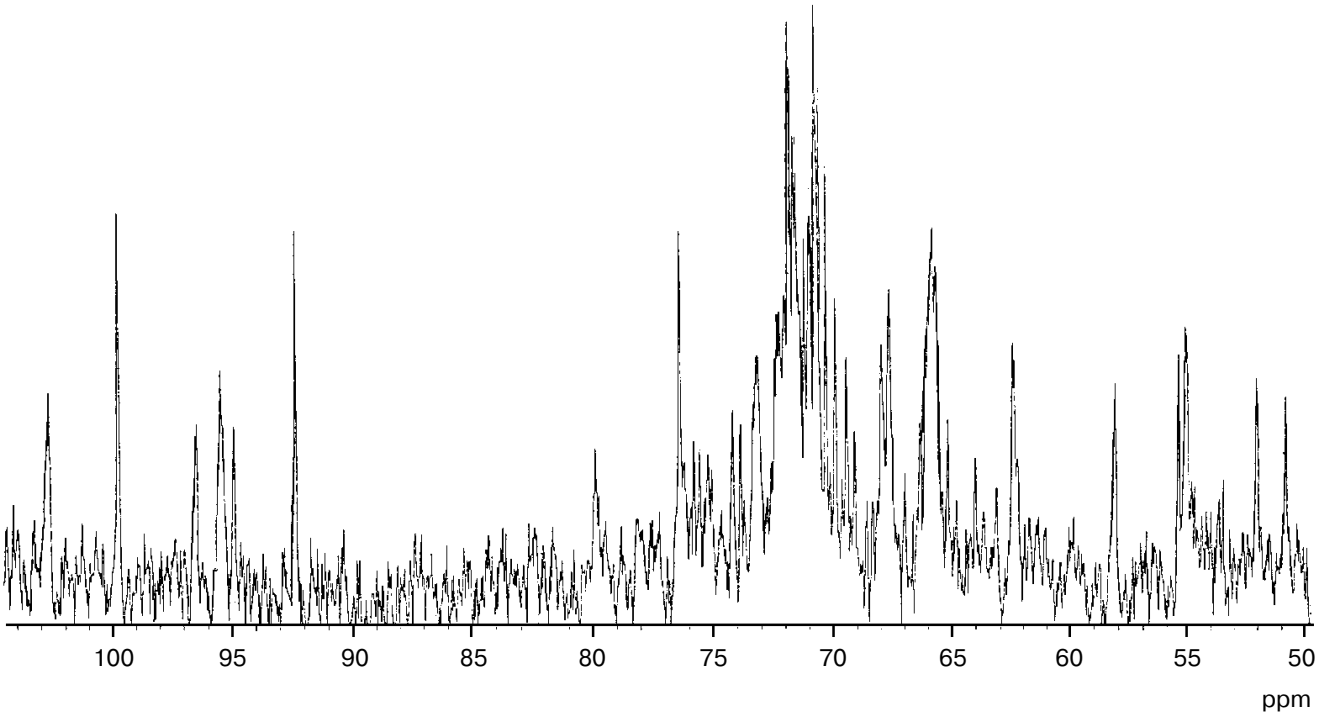
Hydrolysis of the total preparation (0.1 M HCl, 10 min, 100°C) yielded various amount of phosphorous-containing compounds. A compound with mobility m_{GroP} = 0.79 (polymer 1) prevailed. A compound with mobility m_{GroP} = 0.47 was likely to be partially hydrolyzed, and its amount decreased (polymer 2). Galactose-containing phosphoric ester, glucosamine, and phosphorus were also formed. When the time of hydrolysis was increased to 1 h, the amount of this ester also increased. The behavior of polymer 2 on acidic hydrolysis suggested the presence of sugar-1-phosphate bonds [11].

Acidic and alkaline hydrolysis (1 M NaOH, 3 h, 100°C) of polymer 1 yielded glycerol and its mono- and diphosphates; consequently, it was poly(glycerol phosphate). Its content was low, and for this reason we failed to determine by chemical analysis whether polymer 1 is of 1,3- or 2,3-type [12].

On long incubation of the total preparation in aqueous solution (pH 2), polymer 2 was subjected to autohydrolysis; chromatography on a column with a TSK gel gave two fractions, oligomeric (A) and polymeric (B).

Acidic hydrolysis (2 M HCl, 3 h, 100°C) of polymer 3 without phosphorus yielded only galactosamine.

The total preparation and fractions were investigated by NMR spectroscopy. It was noted that the intensity of some signals in ^1H,^{13}C- and ^{31}P-NMR spectra changed with time. The ^{13}C-NMR spectrum of preparation accumulated for 10 h at 30°C is presented in the figure. Six signals of various intensity are in the resonance area of anomeric carbon atoms (δ 102.7, 99.75, 96.45, 95.45, 94.9, and 92.25 ppm). In the spectrum recorded after several days, the intensity of signal at δ 94.45 ppm significantly decreased, whereas intensities of signals at δ 96.45 and 92.25 ppm proportionally increased. After incubation of the sample for a month at room temperature, the signal at δ 95.45 ppm almost disappeared. These data suggest that a labile polymer susceptible to autohydrolysis was present in the preparation; this determined the line of NMR studies. For a freshly dissolved preparation, the 1D



^{13}C-NMR spectrum of cell wall polymers of *S. thermoviolaceus* subsp. *thermoviolaceus* VKM Ac-1857^{T} accumulated for 10 h at 30°C (partial hydrolysis of disaccharide-1-phosphate polymer)

(^{1}H and ^{13}C) and 2D (COSY, TOCSY, ROESY, and HSQC) spectra were recorded.

All these spectra were also recorded for oligomeric (A) and polymeric (B) fractions. In the ^{13}C-NMR spectrum of fraction A, there were observed three signals with various intensity (δ 99.75, 96.45, and 92.25 ppm) in the resonance area of anomeric carbon atoms, two signals (δ 55.4 and 58.0 ppm) in the resonance area of carbon atoms bound to a nitrogen atom, and two pairs of CH_3CON signals (δ 23.4, 23.5 and 175.6, 175.8 ppm for the methyl and carbonyl groups, respectively). In the ^{1}H-NMR spectrum, there were four doublets in the anomeric resonance area: at δ 4.98 and 4.985 ($^{3}J_{1,2}$ 4.0 Hz), 5.23 (4.0 Hz), and 4.75 (8.5 Hz), the total integral intensity of the latter two signals being in correspondence with intensity of the former two signals. The ^{1}H-NMR spectrum of fraction A was attributed by 2D COSY, TOCSY, and ROESY spectra (Table 1). Analysis of the values of spin-coupling constants gave one residue with α-galactopyranose configuration with anomeric protons at δ 4.98 and 4.985 ppm and residues with α- (anomeric proton at δ 5.23 ppm) and β-glucopyranose configuration (anomeric proton at δ 4.75 ppm). In the ROESY spectrum, correlation peaks of the anomeric proton of the residue with *galacto*-configuration and the H-6 and H-6′ protons of the residues with *gluco*-configuration were observed; this indicates that there is a 1→6 bond between them. The HSQC spectrum allowed attributing the carbon atom signals (Table 2). Analysis of the ^{13}C chemical shifts indicated that the residues with *gluco*-configuration are 2-acetamido-2-deoxy-α- and β-glucopyranoses with free hydroxyl group at C-1. A low-field chemical shift for C-6 (δ 67.5 ppm) in the residues proved the presence of a substituent at this position. Comparison of chemical shifts in the ^{13}C-NMR spectra of the residue with the *galacto*-configuration with those of methyl-α-galactopyranoside showed the good coincidence for the C-2, C-3, and C-4 resonances, but different chemical shifts of C-5 and C-6. These differences correspond with the effect of substitution by the phosphate group at C-6 of galactopyranose [13]. The ^{31}P-NMR spectrum proved the presence of phosphate group in the preparation (δ 1.93 ppm) and its localization at C-6 of the galactopyranose residue was proved by the ^{1}H,^{31}P HMQC spectrum, in which correlation peaks of the H-6 protons with phosphorous atoms were observed. So, the oligosaccharide fraction (A) contained disaccharide of the following structure: P-6)-α-Gal*p*-(1→6)-α,β-Glc*p*NAc.

Analogous NMR-spectroscopic study of fraction B showed that it contains two polymers; one of these polymers was identified as 1,3-poly(glycerol phosphate) by the ^{1}H, ^{13}C, and ^{31}P spectra (Tables 1 and 2, polymer 1).

As for the second polymer, in the ^{13}C-NMR spectrum two signals in the resonance area of anomeric carbon atoms at δ 94.9 and 102.7 ppm, two signals of carbon atoms bound to a nitrogen atom (δ 50.8 and 52.1 ppm), and two pairs of CH_3CON signals (δ 23.3, 23.6 and 175.4, 175.9 ppm for the methyl and carbonyl groups, respectively) were found. The attached-protons test (APT) [14]

Table 1. Chemical shifts in the ^{1}H-NMR spectra of carbohydrate-containing polymers of cell wall of *S. thermoviolaceus* subsp. *thermoviolaceus* VKM Ac-1857^T. Chemical shifts of the methyl protons of N-acetyl groups are δ 1.99-2.06

Residue	Chemical shifts, δ (δ_H TSP 0.0)						
Polymer 1	H-1.3	H-2	H-1.3				
-1)-sn-Gro-(3-P-	3.93	4.04	3.87				
Dimers and polymer 2	H-1	H-2	H-3	H-4	H-5	H-6	H-6'
P-6)-α-Galp-(1→	4.98*	3.83	3.93	4.10	4.12	3.95	3.89
	4.985**						
→6)-α-Glc*p*NAc	5.23	3.90	3.75	3.61	4.02	4.04	3.75
→6)-β-Glc*p*NAc	4.75	3.68	3.54	3.61	3.63	4.01	3.81
-6)-α-Gal*p*-(1→	4.98	3.83	3.93	4.05	4.14	4.13	4.03
→6)-α-Glc*p*NAc-(1-P-	5.47	3.89	3.75	3.59	4.02	4.02	3.73
Polymer 3							
→6)-α-Gal*p*NAc-(1→	5.07	4.225	3.80	4.05	3.96	3.93	3.78
→3)-β-Gal*p*NAc-(1→	4.58	4.075	3.82	4.11	3.66	3.82	3.76

* Residue bound to α-Glc*p*NAc.
** Residue bound to β-Glc*p*NAc.

Table 2. Chemical shifts in the ^{13}C-NMR spectra of carbohydrate-containing polymers of cell wall of *S. thermoviolaceus* subsp. *thermoviolaceus* VKM Ac-1857^T. Chemical shifts of the carbon atoms of N-acetyl groups are δ 23.1-23.8 (CH_3) and 175.1-176.8 (CO)

Residue	Chemical shifts, δ (δ_C acetone 31.45)					
	C-1	C-2	C-3	C-4	C-5	C-6
Polymer 1						
-1)-sn-Gro-(3-P-	67.5	70.3	67.5			
Dimers and polymer 2						
P-6)-α-Galp-(1→	99.75	69.9	70.7	70.1	71.1	64.1
→6)-α-Glc*p*NAc	92.25	55.4	72.4	71.3	71.7	67.5
→6)-β-Glc*p*NAc	96.45	58.0	75.3	71.2	75.8	67.5
-6)-α-Gal*p*-(1→	99.75	69.9	70.7	70.2	71.0	65.9
→6)-α-Glc*p*NAc-(1-P-	95.45	55.1	72.4	71.4	71.7	67.5
Polymer 3						
→6)-α-Gal*p*NAc-(1→	94.9	50.8	68.7	69.1	71.4	71.6
→3)-β-Gal*p*NAc-(1→	102.7	52.1	76.1	65.2	76.1	62.5

revealed the signals of one free (δ 62.5 ppm) and one substituted (δ 71.6 ppm) hydroxymethyl group.

In the ^{1}H-NMR spectrum of this fraction signals of two anomeric protons at δ 5.07 ppm (doublet, 4 Hz) and 4.58 ppm (doublet, 8.5 Hz) were observed. Analysis of the COSY, TOCSY, and ROESY spectra showed that the repeating units of polymer 3 are residues of 2-acetamido-2-deoxy-α- and β-galactopyranoses. The linear sequence

of the residues was determined by analysis of the ROESY spectrum, in which correlation peaks of the anomeric proton of the β-Gal*p*NAc residue with the H-6 and H-6′ protons of the α-Gal*p*NAc residue and the anomeric proton of the α-Gal*p*NAc residue with the H-3 and H-4 protons of the β-Gal*p* residue were observed. The signals in the ^{13}C-NMR spectrum were attributed by analysis of the 2D ^{1}H,^{13}C HSQC spectrum, and the substitution of the α-Gal*p*NAc residue at position 6 and the β-Gal*p*NAc residue at position 3 was proved. So, the second polymer in fraction B appeared to be a neutral polysaccharide (polymer 3) with the repeating unit: →6)-α-Gal*p*NAc-(1→3)-β-Gal*p*NAc-(1→.

Analyzing the 1D and 2D spectra of the total preparation, all peaks of the fraction A disaccharide and fraction B polymers were found. The remaining signals appeared to belong to a labile polymer (polymer 2); as a result of its autohydrolysis, a disaccharide is obtained: P-6)-α-Gal*p*-(1→6)-α,β-Glc*p*NAc. The initial structure of the repeating unit of this polymer was found as -6)-α-Gal*p*-(1→6)-α-Glc*p*NAc-(1-P- by analysis of its subspectra in the spectrum of the total preparation.

So, the cell wall of *S. thermoviolaceus* subsp. *thermoviolaceus* VKM Ac-1857^{T} contains two anionic carbohydrate-containing polymers and a polysaccharide. One of anionic polymers is teichoic acid of 1,3-poly(glycerol phosphate) nature (polymer 1), another is disaccharide-1-phosphate polymer with the repeating unit: -6)-α-Gal*p*-(1→6)-α-Glc*p*NAc-P- (polymer 2). The repeating unit of the polysaccharide is the disaccharide: →6)-α-Glc*p*NAc-(1→3)-β-Gal*p*NAc-(1→ (polymer 3). The integral intensity of signals in the resonance area of anomeric carbon atoms indicated that the disaccharide-1-phosphate polymer quantitatively prevails. Its content corresponds to the total content of the two other polymers, polysaccharide and 1,3-poly(glycerol phosphate), which are present in equimolar ratio.

The detection of lysine and its amide and hydroxamate in the cell wall and lysine in the degradation products of the total polymer preparation indicates that lysine is bound to the polymer chains with an ester bond, as was found earlier for other streptomycetes [10].

The data indicate that the cell wall of a strain of a typical species of thermophilic streptomycete, *S. thermoviolaceus*, contains anionic polymers of various nature: teichoic acid — 1,3-poly(glycerol phosphate), disaccharide-1-phosphate polymer, and also polysaccharide without phosphorus. Thus, the wide structural variety of these natural polymers and their combinations in the same cell wall is once more demonstrated. It should be noted that along with polymers widely present in cell walls of gram-positive bacteria, the cell wall of the studied thermophilic streptomycete contains polymers with previously unknown structures.

1,3-Poly(glycerol phosphate) chains, both unsubstituted and containing glycosyl substituents (glucose, galactose, disaccharides, and aminosaccharides), are the teichoic acids most widespread among gram-positive bacteria [12]. However, cell walls of actinomycetes with the same set of anomeric polymers have not been found [1, 5, 12, 15-17].

It was also found that *S. thermoviolaceus* cell wall contains polymers with structures not described earlier: disaccharide-1-phosphate (compounds of this type are rarely found in cell walls of gram-positive bacteria [4, 18] and their structure is not always completely established) [12] and polysaccharide without phosphorus. The latter compounds are found only in some actinomycetes [3, 5, 19].

Genetic studies [7] and the structure of cell wall anionic polymers indicate that *S. thermoviolaceus* subsp. *thermoviolaceus* VKM Ac-1857^{T} streptomycete is most likely a separate species and that the structure of cell surface polymers can serve as an additional chemotaxonomic marker on determination of bacteria species.

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