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ORIGINAL PAPER

Cell wall glycopolymers of *Streptomyces albus*, *Streptomyces albidoflavus* and *Streptomyces pathocidini*

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Abstract The cell wall glycopolymers of three strains of *Streptomyces albus* and the type strain of *Streptomyces pathocidini* were investigated. The structures of the glycopolymers were established using a combination of chemical and NMR spectroscopic methods. The cell wall of *S. albus* subsp. *albus* VKM Ac-35^T was found to be comprised of three glycopolymers, viz. unsubstituted 1,5-poly(ribitol phosphate), 1,3-poly(glycerol phosphate) substituted with β-D-glucopyranose, and the major polymer, a 3-deoxy-D-glycero-D-galacto-non-2-ulosonic acid (Kdn)-teichulosonic acid: β-D-Glc*p*-(1 → 8)-α-Kdn*p*-(2[(→6)-β-D-Glc*p*-(1 → 8)-α-Kdn*p*-(2 →]$_n$6)-β-D-Glc*p*-(1 → 8)-β-Kdn*p*-(2-OH, where $n \geq 3$. The cell walls of '*S. albus*' J1074 and '*S. albus*' R1-100 were found to contain three glycopolymers of identical structures, viz. unsubstituted 1,3- and 2,3-poly(glycerol phosphates), and the major polymer, a Kdn-teichulosonic acid with an unusual structure that has not been previously described: β-D-Gal*p*-(1 → 9)-α-Kdn*p*-(2[(→3)-β-D-Gal*p*-(1 → 9)-α-Kdn*p*-(2 →]$_n$3)-β-D-Gal*p*-(1 → 9)-β-Kdn*p*-(2-OH, where $n \sim 7$–8. The cell wall of *S. pathocidini* (formerly *S. albus* subsp. *pathocidicus*) VKM Ac-598^T was found to contain two glycopolymers, viz. 1,3-poly(glycerol phosphate) partially O-glycosylated with 2-acetamido-2-deoxy-α-D-glucopyranose and/or *O*-acylated with L-lysine, and a poly(diglycosyl 1-phosphate) of hitherto unknown structure: -6)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*NAc-(1-*P*-.

Keywords *Streptomyces* · Cell wall · Poly(glycosyl 1-phosphate) · Teichulosonic acid · Teichoic acid · NMR-spectroscopy

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Introduction

Members of the genus *Streptomyces* (class Actinobacteria, suborder *Streptomycineae*, family *Streptomycetaceae*) are Gram-positive bacteria with high mol% G + C of their DNA that produce filamentous branching vegetative and aerial hyphae bearing long chains of reproductive spores; these bacteria are characterised by a complex life cycle of morphological differentiation (Kämpfer 2006). They are widely distributed in nature, especially in soils throughout the world. Streptomycetes demonstrate diverse physiological and metabolic properties and synthesise a large number of secondary metabolites such as antibiotics and immunosuppressants, as well as antifungal, antitumour, antiviral, and antiparasitic agents (Olano et al. 2014; Harrison and Studholme 2014), which play an important role in medicine, industry, and agriculture. The type species of the genus *Streptomyces* is *Streptomyces albus*, which is notable for the ability to produce mutants that are used for applied and scientific purposes (Chater and Wilde 1980).

In recent years, special attention has been given to study of the genomes of streptomycetes to search for gene clusters for biosynthesis of secondary metabolites, in particular antibiotics of new structures and functions hitherto not used in medicine (Doroghazi and Metcalf 2013). However, success in this regard requires knowledge of the biology of potential producers of secondary metabolites. The study of their cell wall glycopolymers gives understanding of the compounds in the cell envelopes of streptomycetes. These polymers have a number of important physiological functions (Rautenberg et al. 2010; Brown et al. 2013; Petrus and Claessen 2014). They play an important role in the mechanisms of interactions of the bacteria within the microbial community and the environment, including higher organisms, and they can define the immune properties of microorganisms. Our previous studies have revealed a great diversity of anionic glycopolymers in the cell walls of *Streptomyces* species: among them were found teichoic acids (TAs), teichuronic acids (TUAs), teichulosonic acids (TULAs), glycosyl 1-phosphates (GPs) and polysaccharides (PSs) (Shashkov et al. 2002, 2006; Kozlova et al. 2006; Streshinskaya et al. 2007; Tul'skaya et al. 2007a, 2011).

In this work, the structure and composition of the cell wall glycopolymers of some representatives of the cluster *S. albus*: *S. albus* subsp. *albus* VKM Ac-35^{T}, *S. albus* subsp. *pathocidicus* VKM Ac-598^{T}, as well as '*S. albus*' J1074 and '*S. albus*' R1-100, were studied for the first time. It is noted that, based on 16S rRNA sequence analysis by Labeda et al. (2012), *S. albus* subsp. *albus* VKM Ac-35^{T} was recovered in cluster 126, whereas *S. albus* subsp. *pathocidicus* VKM Ac-598^{T} was recovered in cluster 120. Subsequently, this subspecies has been reclassified as a separate species, *Streptomyces pathocidini* (Labeda et al. 2014). *S. albus* strain NBRC 1304^{T} (VKM Ac-35^{T}) has been genome sequenced (Komaki et al. 2015). '*S. albus*' J1074 is a derivative of '*S. albus*' G that is defective in the SalIG1 restriction–modification system, has a valine–isoleucine auxotrophic phenotype (Chater and Wilde 1976) and is sensitive to the antibiotic moenomycin A. This strain is characterised by very fast and dispersed growth, simplicity of genetic manipulations, has the smallest known genome among the representatives of the genus *Streptomyces* and is used for heterologous production of bioactive natural products (Olano et al. 2014; Zaburannyi et al. 2014; Myronovskyi et al. 2014; Seipke 2015). Based on multilocus sequence analysis, Labeda et al. (2014) concluded that strain J1074 is misidentified and in fact should be classified as a strain of *Streptomyces albidoflavus* (clade 112 defined by Labeda et al. 2012). '*S. albus*' R1-100 is a spontaneous moenomycin A-resistant derivative of '*S. albus*' J1074. The sensitivity or resistance to moenomycin A of a microorganism presumably depends upon the structural features of its cell envelope, such as the presence of specific glycopolymers, since the antibiotic targets peptidoglycan glycosyltransferase activities (Ostash and Walker 2010).

Materials and methods

Strains and culture conditions

Streptomyces albus VKM Ac-35^{T} (=DSM 40313^{T} = NRRL B-2208^{T} = NBRC 13014^{T}) and *S. pathocidini* VKM Ac-598^{T} (=DSM 40799^{T} = NRRL B-24287^{T} = NBRC 13812^{T}; formerly *S. albus* subsp. *pathocidicus*) were obtained from the All Russian Collection of Microorganisms (VKM), Skryabin Institute of Biochemistry and Physiology of Microorganisms of the Russian Academy of Sciences. '*S. albus*' J1074 and '*S. albus*' R1-100 are maintained in the collection of microorganisms of Ivan Franko National University of Lviv (http://lv-microbcollect.lviv.ua).

Biomass of the above mentioned streptomycetes was accumulated by growing cultures aerobically in a liquid peptone–yeast medium to the middle of the exponential phase in shaking flasks at 28 °C as described earlier (Potekhina et al. 2011). The mycelium was harvested by centrifugation, washed with 0.95 % NaCl, stored at −18 °C, and used for preparation of the cell walls.

Preparation of cell walls and extraction of glycopolymers

Native cell walls were obtained from crude mycelium by fractional centrifugation after preliminary disruption by sonication in ice water (UP100H, Hielscher, Germany, 30 kHz) and purified using 2 % sodium dodecyl sulfate to avoid possible contamination with membrane components, including lipoteichoic acids, washed several times with water, and lyophilised.

Glycopolymers were isolated from the cell walls by various extraction methods to obtain preparations enriched in particular polymers: (1) the glycopolymer preparations (*preparation* 1) were isolated from cell walls with 10 % trichloroacetic acid at 2–4 °C by three successive extractions for 24, 48, and 72 h; the extracts were separated from cell debris, combined, dialysed against distilled water, and lyophilised; (2) the glycopolymer preparations (*preparation* 2) were isolated from cell walls with 0.05 M NaOH–glycine buffer (pH 8.2–8.8) at 2–4 °C by two successive extractions for 24 h; the extracts were separated from cell debris, combined, dialysed against distilled water, and lyophilised.

Determination of primary structures and analytical procedures

Acid hydrolysis of the cell walls and preparation of glycopolymers, dephosphorylation of Preparation 1, determination of glycopolymer phosphorus, primary structural determination, and other analytical procedures have been described previously (Potekhina et al. 2011). Ammonia lysis of TAs was carried out as described earlier (Streshinskaya et al. 1981).

Chromatography and electrophoresis

Descending paper chromatography and electrophoresis were carried out on Filtrak FN-3 paper (Germany) using various solvent systems. Molybdate reagent was used for detection of phosphate-containing compounds and zones of native glycopolymers; ninhydrin for detection of aminosugars, lysine, and its amide; 5 % $AgNO_3$ in aqueous ammonia for detection of polyols, monosaccharides, and glycosides; aniline hydrogen phthalate reagent for detection of reducing sugars. All procedures were carried out as described earlier (Potekhina et al. 2011).

Determination of absolute configurations

The absolute configurations of six-carbon sugars were determined by GLC following their conversion into acetylated (*S*)-octan-2-yl (α- and β-Gal*p*) or (*S*)-butan-2-yl (α-Glc*p*NAc) derivatives and comparison with reference samples (Gerwig et al. 1979). That of lysine was determined as described earlier (Shashkov et al. 2006).

NMR spectroscopy

The NMR spectra were recorded using a Bruker Avance 600 spectrometer for solutions in 99.96 % D_2O at 30 °C. TSP (δ_H 0.0 and δ_C −1.6) was used as internal standard for the ^1H and ^{13}C spectra and 85 % H_3PO_4 (δ_P 0.0) as an external standard for ^{31}P spectra. Standard pulse sequences were used for 2D ^1H, ^1H COSY, TOCSY, ROESY, ^1H, ^{13}C HSQC, HMBC, and ^1H, ^{31}P HMBC spectra. A mixing time was set to 100 ms in the TOCSY experiments. A spin-lock time of 150 ms was used in the ROESY experiments. Both ^1H, ^{13}C and ^1H, ^{31}P 2D HMBC experiments were optimised for coupling constants of 8 Hz.

Results

The native cell walls of the streptomycetes under study were obtained from crude mycelium by sonication and fractional centrifugation. All *preparations* 1 from streptomycetes under study (obtained by stepwise extraction of cell walls with trichloroacetic acid) were used to determine the qualitative composition of cell wall glycopolymers and the structure identification of phosphate-containing polymers (TAs and GPs). The 3-deoxy-D-glycero-D-galacto-non-2-ulosonic acid (Kdn)-TULAs were highly unstable in acidic media and are cleaved to the repeating units during the extraction process, during chromatography, and even whilst long-term recording of the NMR spectra. Consequently the *preparations* 2, enriched in Kdn-TULA, were obtained by extraction with NaOH–glycine buffer from the cell walls of *S. albus* strains VKM Ac-35^T, '*S. albus*' J1074 and R1-100 and were used to determine the structures of the polymers. Structures of the glycopolymers (from *preparations* 1 and 2) were established using a combination of chemical and NMR spectroscopic methods. The ^{1}H, ^{13}C, and ^{31}P NMR spectra of all preparations were recorded. One-dimensional NMR spectra were assigned using the two-dimensional techniques ^{1}H,^{1}H COSY, TOCSY, ROESY, ^{1}H, ^{13}C HSQC, HMBC, and ^{1}H, ^{31}P HMBC. The absolute configurations of the six-carbon sugars were D, and of lysine was L.

Streptomyces albus VKM Ac-35^{T}

The preliminary determination of the polymer composition by chemical methods

The cell wall of *S. albus* Ac-35^{T} contained 1.2 % phosphorus in phosphate-containing polymers. The yield of *preparations* 1 and 2 was about 7.7 and 8.8 %, respectively of the cell wall dry mass. These studies used 71 mg of *preparation* 1 and 77 mg of *preparation* 2.

The compositions of acid hydrolysates (2 M HCl, 100 °C, 3 h) of *preparation* 1 and the cell wall itself were found to be qualitatively identical. Hydrolysis afforded the following products: inorganic phosphate, minor amounts of glycerol and its mono- and bisphosphates, ribitol and its mono- and bisphosphates, anhydroribitol phosphate, and glucose.

Dephosphorylation (HF, 4 °C, 24 h) of *preparation* 1 yielded glycerol, ribitol, and a glycoside with mobility R_{Glc} 1.07 (chromatography on paper). The latter stained with $AgNO_3$ and did not stain with aniline phthalate, and under hydrolysis equimolar proportions of glucose and glycerol (Glc:Gro ~ 1:1) were found. Therefore, the glycoside was determined to be glucosyl-(1 → 2)-glycerol as described in Tul'skaya et al. (1993). Presumably a poly(glycerol phosphate) substituted with glucopyranose on O-2 of glycerol as well as poly (ribitol phosphate) was present in *preparation* 1.

Electrophoretic study of native *preparation* 1 led to the formation of two zones that stained in different ways with the molybdate reagent and had different mobilities (m_{GroP} 1.2—blue; m_{GroP} 0.8—grey) that suggested the presence of several polymers in the cell wall of the streptomycete, among which were presumably TAs and TULA (Tul'skaya et al. 2007b). All chemical studies of *preparation* 2 led to similar results.

The NMR spectroscopic determination of the glycopolymer structures

Preparations 1 and 2 were studied by NMR spectroscopy. The ^{31}P NMR spectrum contained several broad signals of phosphate groups, the most intense being at δ_P +1.1 and −0.3 (Fig. 1; Table 1). The ^{13}C and ^{1}H NMR spectra (Fig. 2, the axes: left and top, respectively) showed signals corresponding to the carbon atoms (δ_C 68.0, 72.2, 72.4) and protons (δ_H 3.95, 4.00, 4.10) of the unsubstituted 1,5-poly(ribitol phosphate) chain (Table 1, **R**). Besides, signals belonging to the C-1 and C-3 (δ_C 67.5, 67.8), as well as to the C-2 (δ_C 78.3) carbon atoms of the 1,3-poly(glycerol phosphate) chain glucosylated with β-D-glucopyranose on hydroxyl at C-2 of glycerol (Table 1, **Gro** and **Gl**) were also observed. Intense signals of the terminal residues indicated short chains for both polymers. The results confirmed the previous assumptions.

Since the Kdn-TULA from the *preparation* 1 was destroyed during extraction with trichloroacetic acid to a disaccharide β-D-Glc*p*-(1 → 8)-β-Kdn*p* (Fig. 3, Formula 1), we present the NMR spectroscopic data on *preparation* 2, containing oligosaccharide fractions of Kdn-TULA where α-Kdn*p* dominated. The *preparation* 2 also contained the above TAs (Fig. 2).

The structure of the initial Kdn-polymer was deduced from analysis of sub-spectra relating to the oligomer containing the α-Kdn residues. The high-field region of ^{1}H and ^{13}C NMR spectra (Fig. 2, top; Table 1) showed signals that are characteristic for H-3 and C-3 of Kdn*p* with α-(δ_C 41.0;δ_H 1.67 and 2.62) and β-(δ_C 40.4;δ_H 1.79 and 2.18) glycoside centre configuration. The ratio of H-3 α- and β-Kdn*p* signals was approximately 4:1. Several signals of anomeric carbons of sugar residues (Fig. 2, bottom) and weak signals of quaternary carbon atoms of Kdn-residues were observed in the down-field region (Table 1).

Analysis of NMR spectra allowed us to identify signals of the disaccharide moiety (the repeating unit of Kdn-TULA, Table 1) i.e. →6)-β-D-Glc*p*-(1 → 8)-α-Kdn*p*-(2→. However, there were also minor signals identified with strongly down-field shifted of H-9 and H-9′ (δ_H 4.70 and 4.56, Fig. 2) compared to those belonging to residues of α-Kdn*p* from the repeating

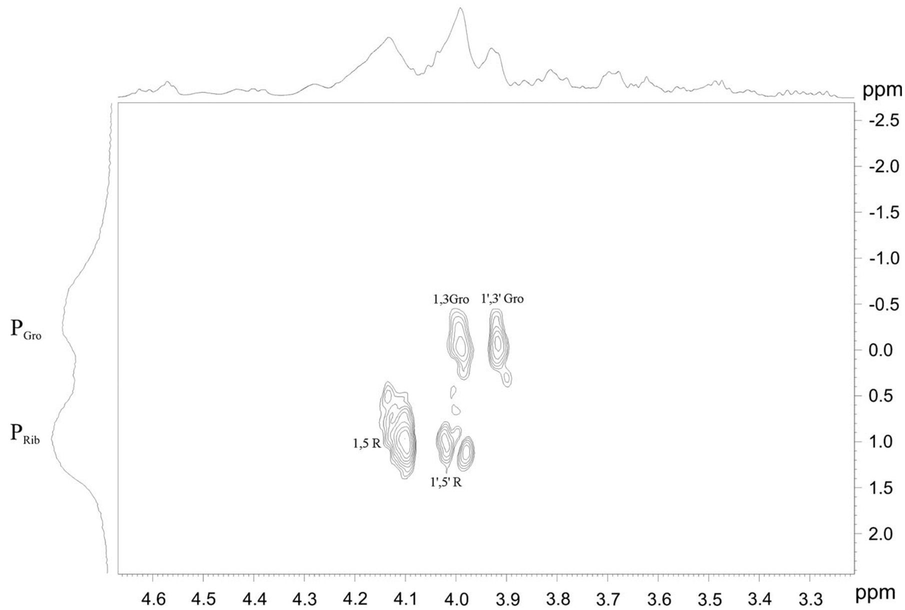

Fig. 1 ^{1}H, ^{31}P HMBC spectrum of teichoic acids (*preparation* 1) from cell wall of *S. albus* VKM Ac-35^T. Arabic numerals refer to the protons in the polyol residues as designated in Table 1. *Gro* glycerol, *R* ribitol

Table 1 ^{13}C and ^{1}H NMR data of the cell wall glycopolymers of *S. albus* VKM Ac-35^T

Polymer residue		Chemical shifts (TSP δ_C -1.6, δ_H 0.00, 85% H_3PO_4 δ_P 0.0)								
		C-1 *H-1,1′*	C-2 *H-2*	C-3 *H-3(eq,ax)*	C-4 *H-4*	C-5 *H-5,5′*	C-6 *H-6,6′*	C-7 *H-7*	C-8 *H-8*	C-9 *H-9,9′*
Teichoic acid I										
-1)-Rib-ol-(5-*P*-	**R**	68.1[a] 4.13, 4.01	71,3 4.16	72.1 3.99	71,3 4.16	68.1[a] 4.13, 4.01				
Theichoic acid II										
-1)-snGro-(3- 2) ↑	**Gro**	66.0[b] 3.99, 3.92	78.0 4.24	66.0[b] 3.99, 3.92						
β-D-Glcp-(1	**Gl$_I$**	103.6 4.65	74.4 3.27	76.2 3.53	71.7 3.44	77.1 3.53	61.7 3.92, 3.67			
Teichulosonic acid										
→ 6)-β-D-Glc*p*-(1→	**Gl$_{II}$**	104.9 4.63	74.7 3.36	75.7 3.54	70.9 3.42	75.8 3.75	63.9 3.97, 3.72			
→ 8)-α-Kdn-(2 →	**K$_α$**	175.0	102.1	41.0 2.62, 1.67	71.0, 71.6 3.59	76.8 3.46	75.7 3.81	68.0 4.28	86.2 3.97	63.4 3.97, 3.75
Disaccharide										
β-D-Glc*p*-(1→	**Gl$_{III}$**	102.7 4.58	74.7 3.33	76.2 3.53	71.7 3.44	77.1 3.53	61.7 3.92,3.67			
→ 8)-β-Kdn	**K$_β$**	174.3	94.7	40.4 2.18, 1.79	70.5 3.95	71.8 3.57	72.4 3.99	68.1 4.05	79.5 4.00	61.9 3.95, 3.83

[a] δ_P +1.1

[b] δ_P −0.3

unit (δ_H 3.97 and 3.75, Fig. 2). This shift is characteristic of *O*-acylated molecular fragments. We concluded that the cause of this effect is the formation of a intramolecular 1–9 macrocyclic Kdn-lactone during isolation of the polymer (Fig. 3, Formula 2). High-field shifting of the signal C-8 (δ_C 80.0–83.0, δ_H 4.32–4.23) of residues with 1–9 lactone compared with C-8 in fragments →6)-β-D-Glc*p*-(1 → 8)-α-Kdn*p*-(2 → (δ_C 86.2, δ_H 3.97) confirmed our assumption (Fig. 2). The presence of two sets of lactone

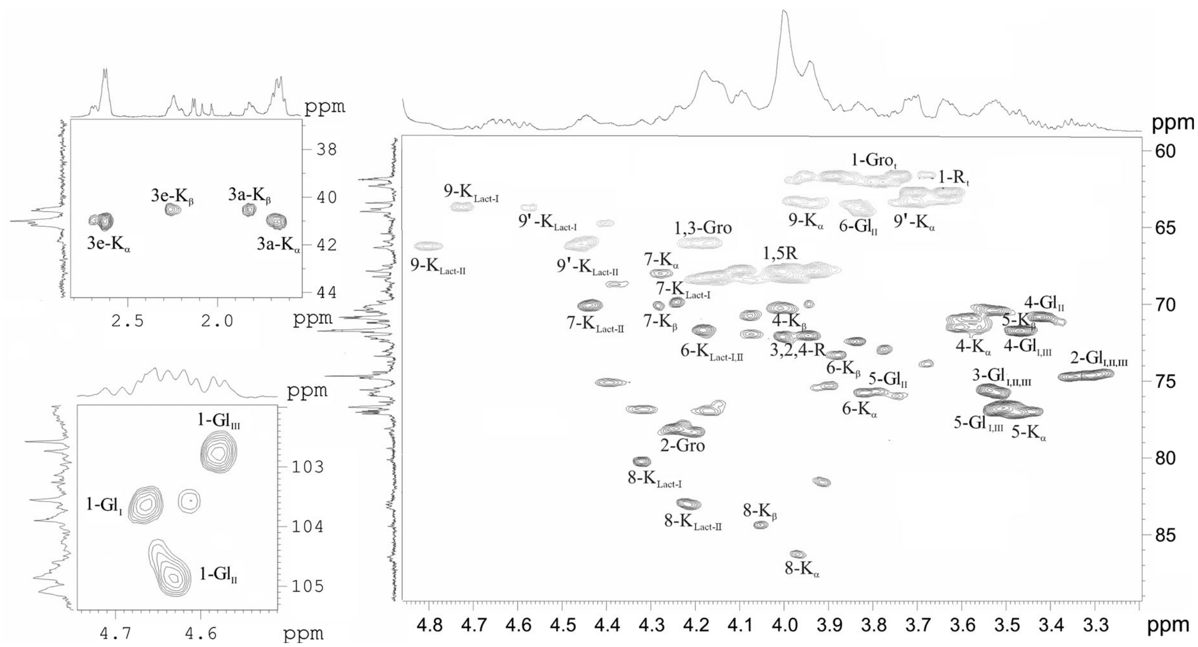

Fig. 2 Parts of [1]H,[13]C HSQC spectrum of glycopolymers (*preparation* 2) from cell wall of *S. albus* VKM Ac-35[T]. Arabic numerals refer to the numbers of atoms in the glycopolymer residues as designated in Table 1. Roman numerals refer to the numbers of glucose residues in Table 1. *Gl* glucopyranose, *Gro* glycerol, *R* ribitol, *K* Kdn-teichulosonic acid, *Lact* lactone

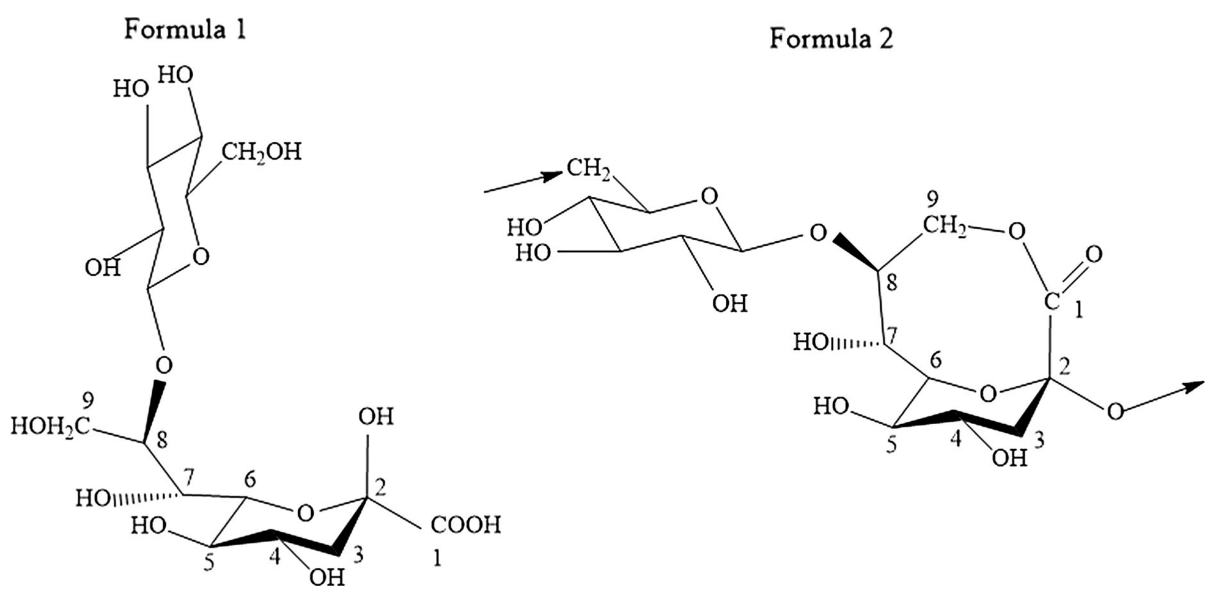

Fig. 3 Structure of the Kdn-teichulosonic acid fragments from the cell wall of *S. albus* VKM Ac-35[T]: disaccharide, the final degradation product of Kdn-teichulosonic acid (Formula 1), and its intramolecular 1–9 macrocyclic Kdn-lactone (Formula 2)

signals (Lact-I and Lact-II, Fig. 2) might be explained if there are two stable cyclic conformers of eight-members of the same 1–9 lactone. Attempts to uncover the lactone macrocycle led to the formation of the disaccharide β-D-Glc*p*-(1 → 8)-β-Kdn*p* (Fig. 3, Formula 1).

In conclusion, the cell wall of *S. albus* Ac-35[T] contained three glycopolymers. Two of them (minor polymers) were the TAs: unsubstituted 1,5-poly(ribitol phosphate) and 1,3-poly(glycerol phosphate) with β-glucopyranose (β-D-Glc*p*) residues at O-2 of most of the glycerol residues. The third glycopolymer was a Kdn-TULA of following structure: β-D-Glc*p*-(1 → 8)-α-Kdn*p*-(2[(→6)-β-D-Glc*p*-(1 → 8)-α-Kdn*p*-(2→]$_n$6)-β-D-Glc*p*-(1 → 8)-β-Kdn*p*-(2-OH, where $n \geq 3$. Taking into account the lability of the Kdn-TULAs, it can be assumed that the length of the native polymer may be greater.

'*S. albus*' J1074 and R1-100 (*S. albidoflavus* strains)

The preliminary determination of the polymers composition by chemical methods

The cell walls of '*S. albus*' J1074 and R1-100 contained 0.8–0.9 % phosphorus in phosphate-containing polymers. The yield of *preparations* 1 and 2 for both organisms was about 6 and 9.5 % of the cell wall dry mass, respectively, 120 and 73 mg of *preparations* 1 and 123 and 76 mg of *preparations* 2, respectively, were studied.

The compositions of acid hydrolysates (2 M HCl, 100 °C, 3 h) of both *preparation* 1 and the cell walls themselves were studied by electrophoresis and chromatography on paper; they were found to be qualitatively identical. Hydrolysis afforded the following products: inorganic phosphate, glycerol and its mono- and bisphosphates, and galactose. These data suggested the presence of poly (glycerol phosphate) in each *preparation* 1. Electrophoretic study of native *preparation* 1 (from both organisms) led to the formation of two zones having different mobilities (m_{GroP} 1.3 and m_{GroP} 0.7), which suggested the presence of several polymers in their cell walls. Similar data were obtained from chemical and electrophoretic studies of native *preparation* 2 (from both organisms).

The NMR spectroscopic determination of the glycopolymer structures

Preparations 1 and 2 were studied separately by NMR spectroscopy. The results showed qualitative identity of the polymers from the investigated strains (Fig. 4a, b).

The ^{13}C and ^{1}H NMR spectra of each *preparation* 1 showed signals corresponding to the carbon atoms of unsubstituted 1,3- and 2,3-poly(glycerol phosphates) at δ_C 67.8 and 70.9 and δ_C 62.2, 76.6 and 66.0, accordingly (Fig. 5; Table 2). The ^{31}P NMR spectrum (not shown) of each *preparation* 1 contained minor signals at δ_P +0.5 and +0.8 (Table 2). Thus unsubstituted 1,3- and 2,3-poly(glycerol phosphates) are found in the cell walls of these two strains.

We did not find a Kdn-TULA structure in *preparation* 1. Consequently the structure of the Kdn-TULA was established by NMR investigation of *preparation* 2. The ^{13}C and ^{1}H NMR spectra (Table 2) of each *preparation* 2 contained signals of different integral intensity in the anomeric carbon resonance region at δ_C 96.4–104.6, including signals for quaternary carbons at δ_C 96.4 and 99.8 characteristic for C-2 of nonulosonic acid (Fig. 6; Table 2). The ^{13}C NMR spectra also contained the signals of CH–CH_2–C group at δ_C 40.0–40.4 characteristic for C-3 of nonulosonic acid (Fig. 4a, b, top left). The ^{1}H NMR spectra of each *preparation* 2 contained signals for anomeric protons at δ_H 4.50 (Fig. 4a, b bottom left; Table 2) and signals for a CH–CH_2–C group at δ_H 2.71 and 1.78 (H-3eq and H-3ax of nonulosonic acid, accordingly, (Fig. 4a, b top left; Table 2).

Based on the analysis of the 1D and 2D homo- and heteronuclear spectra of each *preparation* 2, spin-systems for β-Gal*p*, α- and β-Kdn*p* were identified. A significant difference in the chemical shifts of the H-3eq (δ_H 2.71) and H-3ax (δ_H 1.78) signals ($\Delta\delta_H$ 0.93 ppm, Fig. 6; Table 2) provided evidence favouring the α-configuration of the glycoside center of Kdn*p*.

The ^{1}H, ^{1}H ROESY spectra (not shown) of *preparation* 2 revealed the contacts of anomeric proton H-1 β-Gal*p* (Table 2) with the protons H-9, 9′ of α-Kdn*p* and the proton H-3ax of α-Kdn*p* and H-3 β-Gal*p* in addition to the trivial contacts of the protons of the same residue. Based on the data obtained from the NMR spectra, the following structure of the repeating unit of Kdn-TULA was concluded: →3)-β-D-Gal*p*-(1 → 9)-α-Kdn*p*-(2→.

The ^{1}H,^{13}C HSQC spectrum (Fig. 4a, b) showed that the preparations contained residues of α-Kdn*p* and β-Kdn*p*. This indicates that the above-mentioned polymer from *preparation* 2 was partially cleaved.

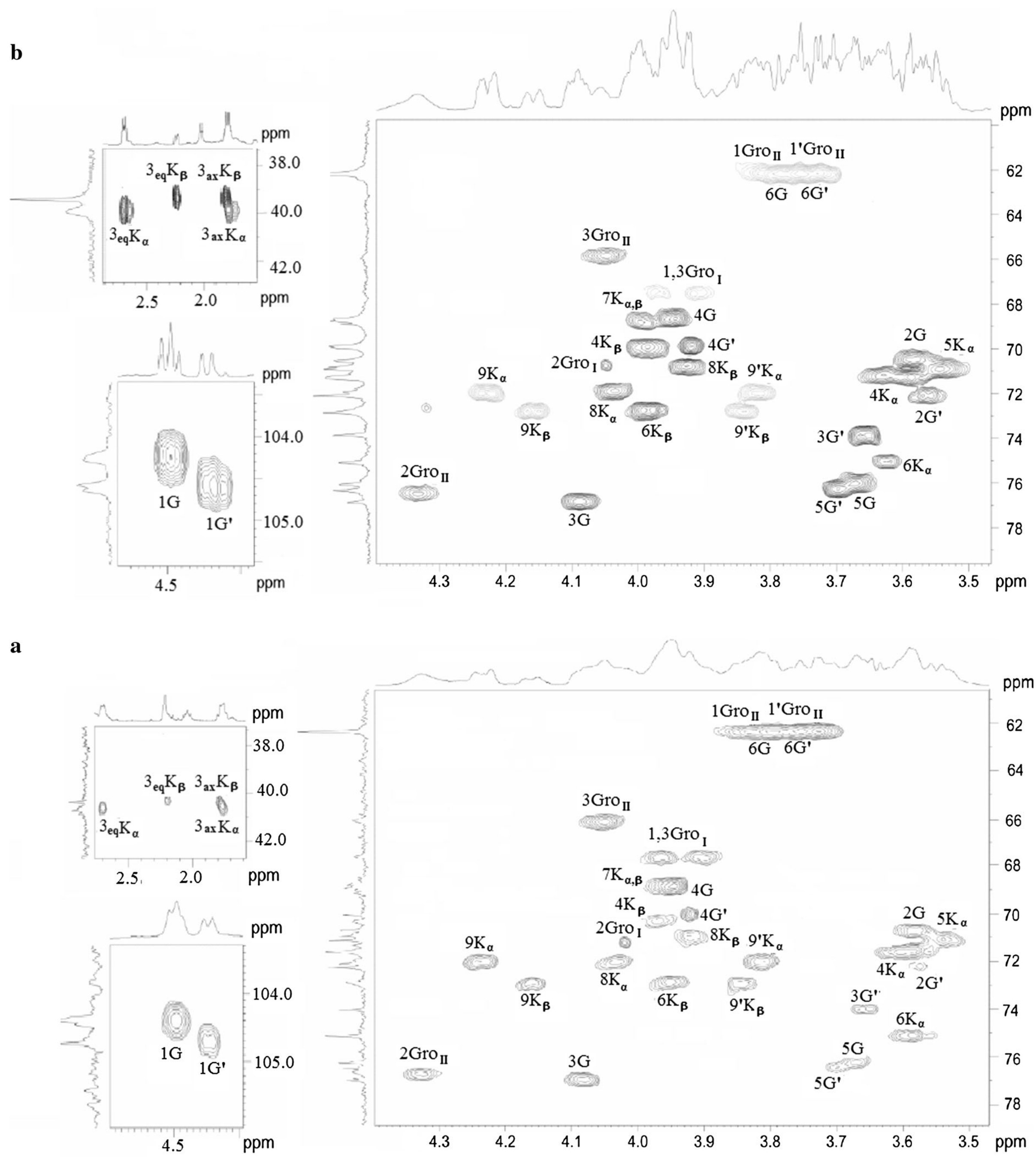

Fig. 4 Parts of [1]H, [13]C HSQC spectra of glycopolymers (*preparation* 2) from cell walls of '*S. albus*' R1-100 (**a**) and J1074 (**b**). Arabic numerals refer to the numbers of atoms in the glycopolymer residues as designated in Table 2. *Gro* glycerol, *G* galactopyranose, *K* Kdn-teichulosonic acid. Roman numerals refer to the numbers of teichoic acids in Table 2

The bond -α-Kdn*p*-(2 → 3)-β-D-Gal*p*-was independently confirmed by the presence of correlation peak 3G/2K_α (δ_H 4.10/δ_C 99.8) in the fairly well-resolved ^{1}H, ^{13}C HMBC spectrum (Fig. 6; Table 2). The spectrum also confirms the β-D-Gal*p*-(1 → 9)-α, β-Kdn*p* bond (Table 2).

In conclusion, the cell walls of '*S. albus*' R1-100 and J1074 contained three glycopolymers: two

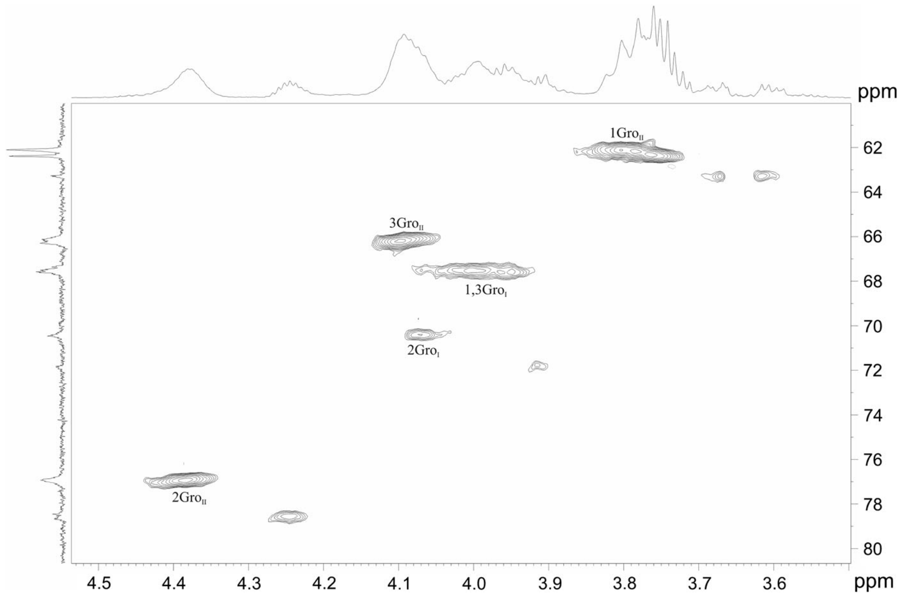

Fig. 5 ^{1}H, ^{13}C HSQC spectrum of teichoic acids (*preparation* 1) from cell walls of '*S. albus*' R1-100. Arabic numerals refer to the numbers of atoms in the glycopolymer residues as designated in Table 2. Roman numerals refer to the numbers of teichoic acids. Abbreviation as Fig. 4

Table 2 ^{13}C and ^{1}H NMR data of the cell wall polymers of '*S. albus*' J1074 and R1-100

Polymer residue	Chemical shifts (TSP δ_C −1.6, δ_H 0.00, 85 % H_3PO_4 δ_P 0.0)								
	C-1 *H-1,1′*	C-2 *H-2*	C-3 *H-3 (eq, ax)*	C-4 *H-4*	C-5 *H-5,5′*	C-6 *H-6,6′*	C-7 *H-7*	C-8 *H-8*	C-9 *H-9,9′*
Teichoic acid I									
→1)-snGro-(3-*P*- **Gro$_I$**	67.8[a]	70.9	67.8[a]						
	4.03, 3.95	4.05	4.03, 3.95						
Teichoic acid II									
-2)-sn-Gro-(3-*P*- **Gro$_{II}$**	62.2	76.6[b]	66.0[b]						
	3.82, 3.75	4.33	4.05, 4.05						
Teichulosonic acid									
→3)-β-D-Gal*p*-(1→ **G**	104.3	70.7	76.9	68.8	76.2	62.4			
	4.50	3.60	4.10	3.95	3.69	3.78, 3.73			
→9)-α-Kdn-(2→ **K$_α$**	173.2	99.8	40.4	71.7	71.1	75.1	68.6	72.1	71.8
			2.71, 1.78	3.62	3.55	3.57	3.98	4.03	4.26, 3.82
Disaccharide from Teichulosonic acid									
β-D-Gal*p*-(1 → **G′**	104.6	72.1	73.9	69.8	76.3	62.2			
	4.43	3.56	3.66	3.92	3.69	3.78, 3.75			
→9)-β-Kdn-(2-OH **K$_β$**	174.2	96.4	40.0	70.0	71.4	72.9	68.8	70.5	72.9
			2.23, 1.80	4.00	3.59	3.99	4.02	3.91	4.15, 3.84

[a] ^{31}P at δ_P +0.5

[b] ^{31}P at δ_P +0.8

TAs i.e. unsubstituted 1,3- and 2,3-poly(glycerol phosphates), and a Kdn-TULA of the following structure: β-D-Gal*p*-(1 → 9)-α-Kdn*p*-(2[(→ 3)-β-D-Gal*p*-(1 → 9)-α-Kdn*p*-(2→]$_n$3)-β-D-Gal*p*-(1 → 9)-β-Kdn*p*-(2-OH, where n ∼ 7–8.

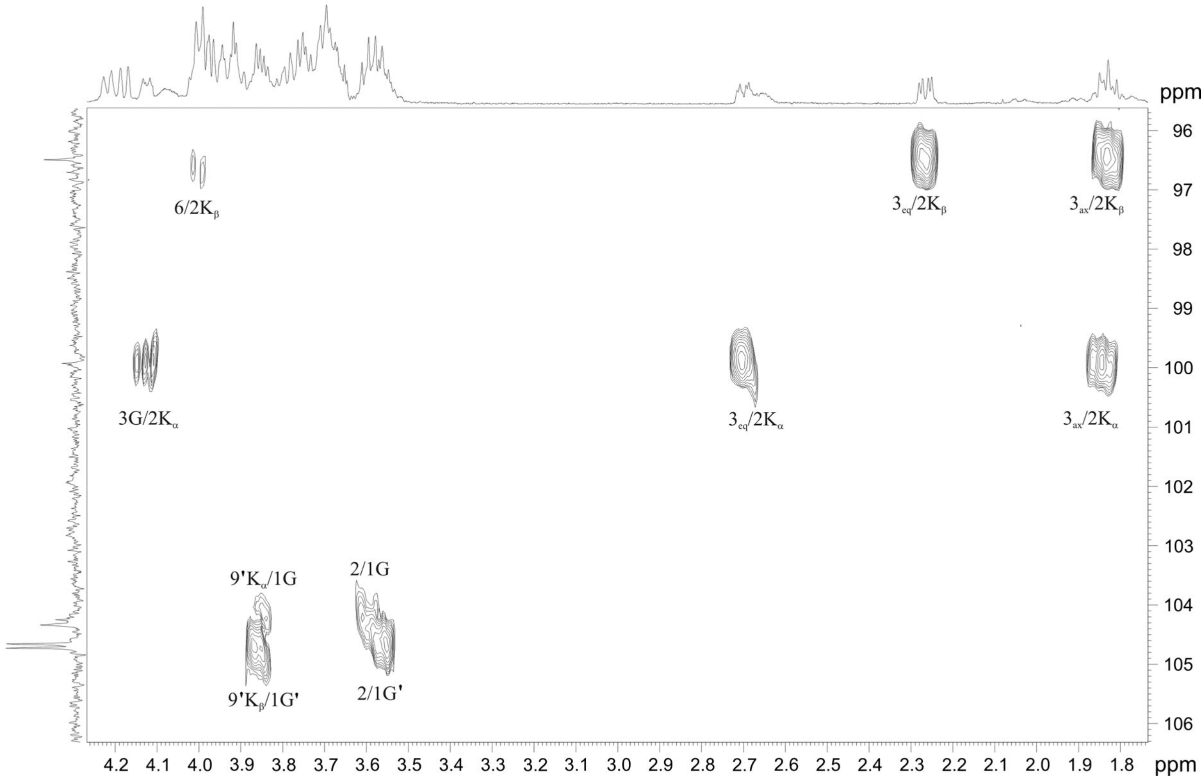

Fig. 6 Part of [1]H, [13]C HMBC spectrum of oligomeric fraction (*preparation* 2) of Kdn-teichulosonic acid from cell wall of '*S. albus*' R1-100. Arabic numerals before slash refer to the protons and after slash refer to carbons in the sugar residues as designated in Table 2

Streptomyces pathocidini VKM Ac-598[T]

The preliminary determination of the polymers composition by chemical methods

The cell wall of this organism contained 2.4 % phosphorus in phosphate-containing polymers. The yield of the *preparation* 1 was about 17.4 % of the cell wall dry mass and was nearly 75 mg. The compositions of acid hydrolysates (2 M HCl, 100 °C, 3 h) of the obtained *preparation* 1 and the cell wall itself were found to be qualitatively identical. Hydrolysis afforded the following products: inorganic phosphate, glycerol, its mono- and bisphosphates, glucosamine, and a small amount of glucose. In addition, lysine was detected in the processing of the cell wall and *preparation* 1 with aqueous ammonia.

Electrophoretic study of the native *preparation* 1 led to the formation of two zones having different mobilities (m_{GroP} 0.6; m_{GroP} 0.4), which suggested the possible presence of two phosphate-containing polymers in the cell wall of this species.

The NMR spectroscopic determination of the glycopolymer structures

The *preparation* 1 was studied by NMR spectroscopy. The [31]P NMR spectrum (not shown) contained two signals of the phosphate groups, the most intense being at δ_P +0.4 and a minor one at δ_P −1.3 (Table 3). These data indicated the possible presence of two different phosphate-containing polymers which agrees with the data of the chemical analysis.

The [13]C NMR spectrum of the *preparation* 1 contained three series of signals (Fig. 7 left axis; Table 3) of different integral intensity: (1) the signals δ_C 67.8 and 70.8 corresponded to the carbon atoms of the unsubstituted 1,3-poly(glycerol phosphate) chain; (2) the signals δ_C 65.1 and 75.5 belonging to residues of glycerol substituted on the C-2 hydroxyl by L-lysine (δ_C 170.5, 54.0, 27.6, 22.7, 30.7, 40.4); (3) the signals δ_C 66.2,66.8 and 77.0 were identified as belonging to residues of glycerol substituted on the hydroxyl at C-2 by α-*N*-acetylglucosamine (δ_C 98.3; 55.0; 72.3; 71.3; 73.5; 61.9; and a signal typical of *N*-acetyl groups

Table 3 ^{13}C and ^{1}H NMR data of the cell wall polymers of *S. pathocidini* VKM Ac-598[T]

Structural fragment		Chemical shifts (TSP δ_C -1.6, δ_H 0.00, 85% H_3PO_4 δ_P 0.0) C-1 *H-1,1′*	 C-2 *H-2*	 C-3 *H-3,3′*	 C-4 *H-4,4′*	 C-5 *H-5*	 C-6 *H-6,6′*
Teichoic acid							
-1)-snGro-(3-*P*-	**Gro$_I$**	67.8[a] 4.00, 3.95	70.5 4.06	67.8[a] 4.00, 3.95			
-1)-snGro-(3-*P*- 2) |	**Gro$_{II}$**	65.1 4.13	75.5 5.41	65.1 4.13			
Lys-(1	**L**	170.5	54.0 4.25	27.6 1.75	22.7 1.60, 1.54	30.7 2.06	40.4 3.04
-1)-snGro-(3-*P*- 2) ↑	**Gro$_{III}$**	66.8 4.09, 4.06	77.0 4.07	66.2 4.10, 4.04			
α-Glc*p*NAc-(1	**GN$_I$**	98.3 5.08	55.0[b] 3.94	72.3 3.79	71.3 3.49	73.5 3.90	61.9 3.87, 3.79
Poly(diglycosyl 1-phosphate)							
(*P*)-6)-α-Glc*p*-(1→	**Gl**	99.4 4.95	73.0 3.56	74.2 3.75	71.1 3.49	72.0 3.85	66.1[c] 4.18, 4.13
→6)-α-Glc*p*NAc-(1-*P*-	**GN$_{II}$**	95.5[c] 5.49	55.1[b] 3.97	72.1 3.73	71.1 3.55	72.0 3.85	67.7 3.95, 3.75

[a] ^{31}P at δ_P +0.4

[b] $\underline{C}H_3\underline{C}ON$ at δ_C 23.4, δ_C 175.7, correspondingly, and δ_H 2.08

[c] ^{31}P at δ_P −1.3

$\underline{C}H_3CON$, δ_C 23.4). These signals are characteristic of a TA of the following structure: 1,3-poly(glycerol phosphate) partially O-glycosylated with 2-acetamido-2-deoxy-α-D-glucopyranose and/or partially *O*-acylated with L-lysine at O-2 of glycerol (Fig. 7; Table 3).

In addition, minor signals belonging to the other phosphate-containing polymer were found. All the above-mentioned data on the composition and structure of compounds in *preparation* 1 and the structure of TA obtained by analysis of its ^{1}H, ^{13}C, and ^{31}P NMR spectra allowed us to establish the structure of the minor phosphate-containing polymer. Analysis of the 2D ^{1}H, ^{13}C HSQC spectrum (Fig. 7; Table 3) demonstrated that the second polymer is built of disaccharide residues α-D-Glc*p*-(1 → 6)-α-D-Glc*p*NAc linked in the polymer chain by phosphodiester bonds between the hydroxyl at C-1 (δ_C 95.5) of α-D-GlcpNAc and hydroxyl at C-6 (δ_C 66.1) of α-D-Glc*p* (Table 3). Based on these data, the structure of the repeating unit of the disaccharide 1-phosphate polymer can be presented as follows: -6)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*NAc-(1-*P*-. All characteristic chemical shifts are presented in Fig. 7 and Table 3. This polymer was partially degraded in the process of extraction and during the long-term recording of the NMR spectra, with cleavage of the C-1–O–P bond. Thus the 2D ^{1}H, ^{13}C HSQC spectrum (Fig. 7, bottom left) contained signals of terminal units at the reducing end of the chain -6)-α-D-Glc*p*NAc-OH and -6)-β-D-Glc*p*NAc-OH. This disaccharide 1-phosphate polymer (GP) is described here for the first time, to our knowledge, in a Gram-positive bacterium.

In conclusion, the cell wall of *S. pathocidini* VKM Ac-598[T] was found to contain two phosphate-containing polymers: a TA—1,3-poly(glycerol phosphate) partially glycosylated with α-D-Glc*p*NAc and/or *O*-acylated with L-lysine at O-2 of glycerol and a GP of following repeating unit -6)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*NAc-(1-*P*-.

Discussion

In this work, the cell wall glycopolymer structures and compositions were identified by chemical and NMR spectroscopic methods. The combination of techniques made it possible to establish the structure of the major polymers as well as to reveal the presence of some different minor polymers in the cell walls of the studied strains.

The cell wall of the type strain *S. albus* VKM Ac-35[T] was found to contain two TAs, viz. unsubstituted 1,5-poly(ribitol phosphate) and 1,3-poly(glycerol phosphate) with β-D-Glc*p* at O-2 of glycerol. This TA was first found in the cell walls of *Streptomyces*

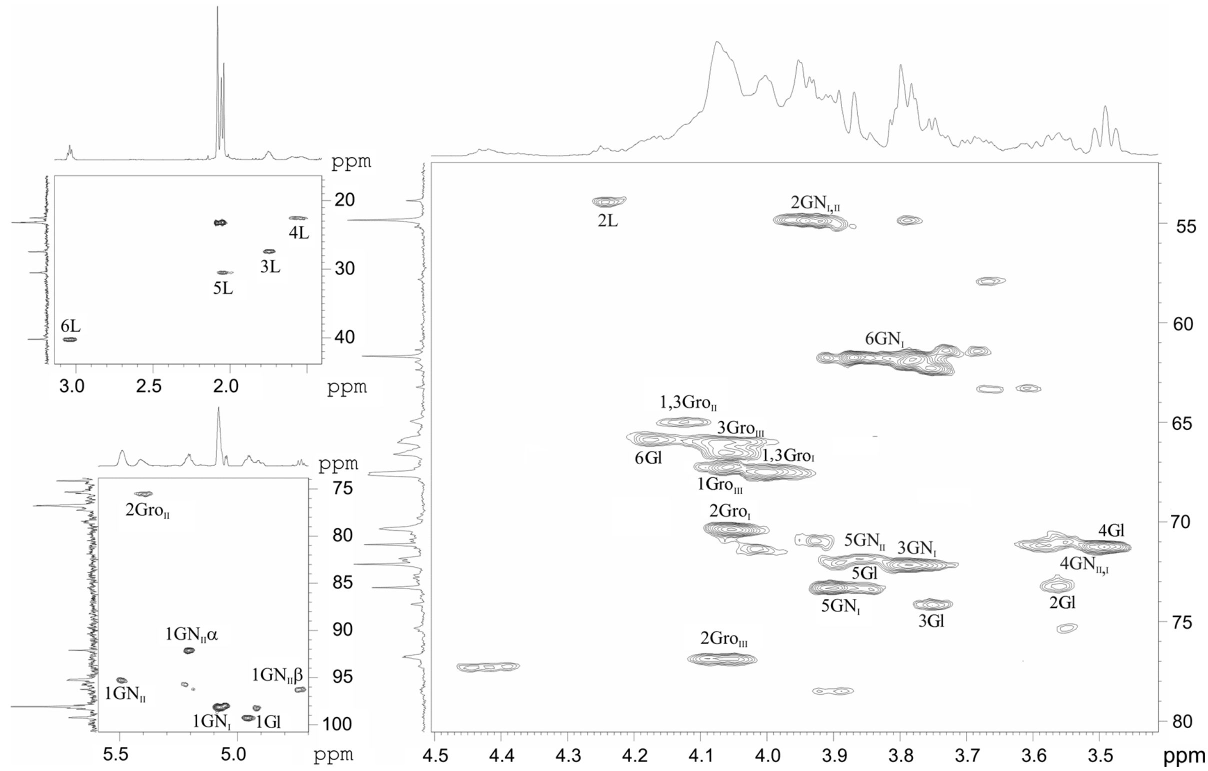

Fig. 7 Parts of [1]H, [13]C HSQC spectrum of glycopolymers (*preparation* 1) from cell walls of *S. pathocidini* VKM Ac-598^T. Arabic numerals refer to the numbers of atoms in the glycopolymer residues as designated in Table 3. Roman numerals refer to the numbers of glycerol residues in teichoic acid and glucosamine residues in both glycopolymers as designated in Table 3. *Gro* glycerol, *L* lysine, *Gl* glucopyranose; *GN* glucosamine

chrysomallus (Streshinskaya et al. 1995), and the second is quite widespread in the cell walls of Gram-positive bacteria (Potekhina et al. 2003; Streshinskaya et al. 2011). The major glycopolymer was identified as a Kdn-TULA with the repeating unit: →6)-β-D-Glc*p*-(1 → 8)-α-Kdn*p*-(2→. Glycopolymers of the same structure were recently found in the cell walls of a number of actinobacteria, viz. *Brevibacterium aurantiacum* VKM Ac-2111^T, *Arthrobacter protophormiae* VKM Ac-2104^T, and *Streptomyces coelicolor* VKM Ac-738^T (Streshinskaya et al. 2015; Shashkov et al. 2015).

Two other strains originally classified as *S. albus*, J1074 and R1-100, differed significantly from the above-described *S. albus* VKM Ac-35^T in composition and structure of cell wall glycopolymers. However, the cell walls of '*S. albus*' J1074 and R1-100 were identical in the composition and structure of their glycopolymers. Among them were unsubstituted 1,3- and 2,3-poly(glycerol phosphates) that commonly occur in streptomycete cell walls (Potekhina et al. 1996; Tul'skaya et al. 1997, 2007b, 2011). The major glycopolymer was a Kdn-TULA with the repeating unit: →3)-β-D-Gal*p*-(1 → 9)-α-Kdn*p*-(2→. In this work, this polymer was found for the first time to our knowledge in a Gram-positive bacterium. The topology of the ketosidic bond (-α-Kdn*p*-(2 → 3)-β-D-Gal*p*) was the main difference between the latter Kdn-TULA compared to that found earlier in *S. coelicolor* M145 (Shashkov et al. 2012).

The cell wall of *S. pathocidini* VKM Ac-598^T was found to contain two phosphate-containing glycopolymers. The major polymer was identified as 1,3-poly(glycerol phosphate) partially O-glycosylated with α-D-Glc*p*NAc and/or partially *O*-acylated with L-lysine at O-2 of glycerol. A TA of this structure was found earlier in the cell walls of a number of streptomycetes (Shashkov et al. 2006; Tul'skaya et al. 2007b). The minor polymer comprised a GP of new structure with the following repeating unit: -6)-α-D-Glc*p*-(1 → 6)-α-D-Glc*p*NAc-(1-*P*-.

Teichoic acids of diverse structures are widespread in the cell walls of streptomycetes (Potekhina et al. 2011; Tul'skaya et al. 2011). In contrast GPs are rarely found in the cell walls of streptomycetes and only a few of such structures have so far described (Kozlova et al. 2006; Potekhina et al. 2011; Shashkov et al. 2012). The Kdn-TULAs were first described in the cell walls of phytopathogenic streptomycetes (Shashkov et al. 2000). Currently, Kdn-TULAs have been found in the cell walls of actinobacteria of different families and genera: *Arthrobacter* sp VKM Ac-2550 and Ac-2549, *A. protophormiae* VKM Ac-2104^T, *B. aurantiacum* VKM Ac-2111^T, *S. albus* strains VKM Ac-35^T, '*S. albus*' J1074 and R1-100, *S. coelicolor* VKM Ac-738^T and *S. coelicolor* M145 (Streshinskaya et al. 2015). A defining component of these polymers is Kdn—a nine-carbon keto-sugar acid 3-deoxy-D-glycero-D-galacto-non-2-ulosonic acid (Tul'skaya et al. 2011). These polymers have a linear structure, and along with Kdn may contain monosaccharides (glucopyranose, galactopyranose, N-acetylglucosamine) in the main chain. In addition, the residues of Kdn may be nonstoichiometrically substituted at O-4 with residues of α-D-Glc*p*NAc/methyl groups (Shashkov et al. 2012). The Kdn-TULAs from the actinomycetes studied to date differ in localisation of the ketosidic and glycosidic bonds.

The Kdn-TULAs are accompanied by various other glycopolymers, viz. TAs and TUAs, GPs, and PSs (Tul'skaya et al. 2011) in the cell walls of all streptomycetes studied up to now. As polyanionic polymers, the Kdn-TULAs can bind cations, impart negative charge to the cell envelope, control autolysin activities, and can be involved in cell communication within the microbial community and the environment, including with higher organisms (Tul'skaya et al. 2011).

Conclusion

This study showed that the related strains '*S. albus*' J1074 and R1-100 (sensitive and resistant, respectively, to moenomycin A, which is a phosphoglycolipid antibiotic that inhibits the biosynthesis of peptidoglycan (Ostash and Walker 2010) contain the same composition and structure of glycopolymers. Thus the sensitivity/resistance to moenomycin A does not depend on the composition and structure of the cell wall glycopolymers. The type strain of *S. albus*, VKM Ac-35^T, was found to have a distinct profile of cell wall glycopolymers. These data support the conclusion that '*S. albus*' J1074 and R1-100 belong to a distinct species, *S. albidoflavus* (Labeda et al. 2014). Likewise, the reclassification of *S. albus* subsp. *pathocidicus* VKM Ac-598^T as a novel species, *S. pathocidini* VKM Ac-598^T (Labeda et al. 2014) is supported by the differences between its cell wall glycopolymer profile and that of *S. albus* VKM Ac-35^T. Taking into account all the data discussed above, the value of determining the structure and composition of cell wall glycopolymers for the taxonomy and species specificity of the members of the genus *Streptomyces* becomes evident.

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