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Structure of a Peptidoglycan-Related Polysaccharide from *Providencia alcalifaciens* O45

O. G. Ovchinnikova[1,2]*, B. Liu[2], N. A. Kocharova[1], A. S. Shashkov[1], A. N. Kondakova[1], M. Siwinska[3], L. Feng[2,4], A. Rozalski[5], L. Wang[2,4], and Y. A. Knirel[1]

[1]*Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Leninsky pr. 47, 119991 Moscow, Russia; fax: (499) 137-6148; E-mail: olga.ovchinnikova@gmail.com*
[2]*TEDA School of Biological Sciences and Biotechnology, Nankai University, TEDA, Tianjin 300457, China; E-mail: striker198126@yahoo.com.cn*
[3]*Department of General Microbiology, Institute of Microbiology, Biotechnology and Immunology, University of Lodz, PL 90-237 Lodz, Poland*
[4]*Tianjin Key Laboratory of Microbial Functional Genomics, Nankai University, TEDA, Tianjin 300457, China*
[5]*Department of Immunobiology of Bacteria, Institute of Microbiology, Biotechnology and Immunology, University of Lodz, PL 90-237 Lodz, Poland*

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Abstract—A polysaccharide was isolated from the opportunistic human pathogen *Providencia alcalifaciens* O45:H26 by extraction with aqueous phenol and studied by sugar and methylation analyses along with ^{1}H and ^{13}C NMR spectroscopy, including two-dimensional ROESY and H-detected ^{1}H,^{13}C HSQC experiments. The polysaccharide contains *N*-acetylglucosamine and *N*-acetylmuramic acid (D-Glc*p*NAc3*R*lac) amidated with L-alanine and has the following structure:

→4)-β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc3(*R*lac-L-Ala)-(1→.

The polysaccharide possesses a remarkable structural similarity to the bacterial cell wall peptidoglycan. It is not unique to the strain studied but is common to strains of at least four *P. alcalifaciens* O-serogroups (O3, O24, O38, and O45). No evidence was obtained that the polysaccharide is associated with the LPS, and hence it might represent a bacterial capsule component.

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Key words: *Providencia alcalifaciens*, lipopolysaccharide, *N*-acetylmuramic acid, structure, bacterial polysaccharide, peptidoglycan

Enterobacteria of the genus *Providencia* are opportunistic human pathogens associated mainly with urinary tract infections and enteric diseases [1]. In particular, *P. alcalifaciens* is significantly associated with diarrhea in travelers and children, although it can be found occasionally in non-diarrheic stools [2, 3]. The combined serological classification scheme of three most medically important species, *P. alcalifaciens*, *P. rustigianii*, and *P. stuartii*, includes 63 O-serogroups and 30 H-serogroups [4]. Recently, it has been found that strains representing serogroups O58:H9 and O59:H18 must be reclassified from *Providencia* to *Morganella morganii* (A. Rozalski, unpublished data).

A peculiar feature of Gram-negative bacteria is the presence of an outer membrane that surrounds a rigid peptidoglycan layer. O-Serotyping of these bacteria is based on the O-antigen, which represents a polysaccharide chain of the lipopolysaccharide (LPS), the key component of the outer membrane. The full LPS molecule

Abbreviations: COSY, correlation spectroscopy; CPS, capsular polysaccharide; ESI MS, electrospray ionization mass spectrometry; GlcN, glucosamine; GlcNAc3*R*lac, *N*-acetylmuramic acid; HSQC, heteronuclear single-quantum coherence; LPS, lipopolysaccharide; *R*lac, (*R*)-lactic acid or (*R*)-1-carboxyethyl group; ROESY, rotating-frame nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; C14:0, C16:0, and C18:0, tetradecanoic, hexadecanoic, and octadecanoic acid; 3OH-C14:0, 3-hydroxytetradecanoic acid.

* To whom correspondence should be addressed.

(S-type) is composed of three structural domains: O-polysaccharide (O-antigen), which is built up of oligosaccharide repeats (O-units), core oligosaccharide, and lipid A, which anchors the LPS molecule into the membrane. The S-type LPS is coexpressed on the cell surface with short-chain LPS species, which either are devoid of any O-polysaccharide (R-type LPS) or possess an O-antigen represented by only one O-unit (SR-type LPS).

Many enteric bacteria also produce another serotype-specific glycopolymer, a capsular polysaccharide (CPS) called in some bacteria as K-antigen [5]. The CPS is a surface-enveloping high-molecular-weight polymer that is bound to the cell usually via a phospholipid anchor.

The O-antigen structures have been established in the majority of *Providencia* O-serogroups [6]. The occurrence of a CPS has been reported in serogroups O14 (two strains), O15, O25, and O46 [7]. However, no further studies have been undertaken to confirm directly the presence of a capsule in these strains or to determine the CPS structures.

Now we report on a new structure of a polysaccharide from *P. alcalifaciens* O45, which is not associated with LPS and, most likely, represents a CPS. It has the shortest repeating unit among *Providencia* polysaccharides studied so far and possesses a remarkable structural similarity to the bacterial cell wall peptidoglycan. A short-chain LPS was isolated from *P. alcalifaciens* O45 too and its composition was determined.

MATERIALS AND METHODS

Providencia alcalifaciens O45:H26 (strain 577/48) obtained from the Hungarian National Collection of Medical Bacteria (National Institute of Hygiene, Budapest) was cultivated under aerobic conditions in tryptic soy broth supplemented with 0.6% yeast extract. The bacterial mass was harvested at the end of the logarithmic growth phase, centrifuged, washed with distilled water, and lyophilized. LPS was isolated in a yield of 2.05% from dried bacterial cells by the phenol–water procedure [8] followed by dialysis of the extract without layer separation, and then freed from insoluble contaminations by centrifugation. The crude LPS was purified by precipitation of proteins and nucleic acids with cold aqueous 50% CCl_3CO_2H at 4°C. After centrifugation, the supernatant was dialyzed against distilled water and freeze-dried.

A portion of the LPS (100 mg) was heated with 2% acetic acid (4 ml) for 13 h at 100°C, and the carbohydrate-containing supernatant was fractionated on a column (60 × 2.5 cm) of Sephadex G-50 superfine in 0.05 M pyridinium acetate buffer, pH 4.5. The yield of the polysaccharide was ~15% of the LPS mass.

For sugar analysis, the polysaccharide was hydrolyzed with 10 M HCl (80°C, 30 min), the products were reduced with an excess of $NaBH_4$ (20°C, 2 h), N,O-acetylated with a 1 : 1 Ac_2O–pyridine mixture (100°C, 1 h), and analyzed by GLC-MS on a Hewlett-Packard HP 5989A instrument (USA) equipped with a 30-m HP-5ms column using a temperature gradient of 150°C (3 min) to 320°C at 5°C/min. Amino components were analyzed on a Biotronik LC-2000 amino acid analyzer (Germany) using standard sodium citrate buffers.

For determination of the absolute configurations of the components [9], the polysaccharide was hydrolyzed as above, the products were N-acetylated (60 μl Ac_2O in 400 μl aqueous saturated solution of $NaHCO_3$, 0°C, 1 h), subjected to (*S*)-2-octanolysis (100 μl (*S*)-2-octanol, 15 μl CF_3CO_2H, 120°C, 16 h), N,O-acetylated as above and analyzed using an Agilent Technologies 7820A GC system (USA) with a HP-5ms column (Agilent) and a temperature gradient of from 160°C (3 min) to 290°C at 7°C/min.

Methylation of the polysaccharide was performed according to the Hakomori procedure [10], and the products were recovered using a Sep-Pak C18 cartridge. Partially methylated monosaccharides were derived by hydrolysis with 10 M HCl (80°C, 30 min), converted into the alditol acetates, and analyzed by GLC-MS as above.

Fatty acids were released by methanolysis of the polysaccharide with 2 M HCl in methanol (85°C, 16 h), extracted with heptane, O-acetylated, and analyzed by GLC-MS using an Agilent MSD 5975 instrument equipped with a HP-5ms column and a temperature gradient from 150°C (3 min) to 320°C at 5°C/min.

ESI MS was performed in the negative ion mode using a micrOTOF II instrument (Bruker Daltonics, Germany). A sample (~50 ng/μl) was dissolved in a 1 : 1 (v/v) water–acetonitrile mixture and sprayed at a flow rate of 3 μl/min. Capillary entrance voltage was set to 4.5 kV and exit voltage to –150 V; drying gas temperature was 180°C.

Samples were freeze-dried twice from a 99.9% D_2O solution and dissolved in 99.96% D_2O. ^1H and ^{13}C NMR spectra were recorded at 30°C using a Bruker DRX-500 spectrometer (Germany). Internal sodium 3-trimethylsilylpropanoate-2,2,3,3-d_4 (δ_H 0) and acetone (δ_C 31.45) were used as references. Mixing times of 100 and 150 msec were used in TOCSY and ROESY experiments, respectively.

RESULTS AND DISCUSSION

A polysaccharide material was obtained from dry bacterial cells of *P. alcalifaciens* O45 by the phenol–water extraction and subjected to mild acid hydrolysis. Lipid precipitate was removed by centrifugation, and the supernatant was fractionated by GPC on Sephadex G-50 to give a high-molecular-mass polysaccharide and two oligosaccharide fractions, A and B.

Sugar analysis of the polysaccharide by GLC-MS of the acetylated alditols derived after complete acid hydrolysis showed the presence of 2-amino-2-deoxyglucose (GlcN) and 2-amino-3-*O*-[(*R*)-1-carboxyethyl]-2-deoxyglucose (GlcN3*R*lac, muramic acid) in the ratio ~1 : 1. Amino acid analysis revealed alanine. The D-configuration of GlcN and the L-configuration of alanine were determined by GLC of the acetylated (+)-2-octyl glycosides and (+)-2-octyl esters, respectively; the D-configuration of GlcN3*R*lac was confirmed by analysis of glycosylation effects on ^{13}C NMR chemical shifts [11] (see below). GLC-MS analysis of the partially methylated alditol acetates derived from the methylated polysaccharide revealed 4-substituted GlcN and 4-substituted GlcN3*R*lac.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 1) contained signals for one alanine and two sugar residues, including those for two anomeric carbons at δ 101.5 and 102.6, three nitrogen-bearing carbons (C-2 of GlcN, GlcN3*R*lac and Ala) at δ 50.4-56.5, two methyl groups (H-3 of Ala and *R*lac) at δ 17.4 and 18.9, two *N*-acetyl groups (CH_3 at δ 23.2 and 23.4, CO at δ 175.4 and 175.7), and two more CO groups (C-1 of Ala and *R*lac) at δ 174.7 and 178.8. There were no signals in the region δ 82-88 and, hence, both sugar residues occur in the pyranose form [12]. The ^{1}H NMR spectrum of the polysaccharide contained *inter alia* signals for two anomeric protons at δ 4.50 and 4.52, two methyl groups (H-3 of Ala and *R*lac) at δ 1.37 and 1.43, and two *N*-acetyl groups at δ 1.98 and 2.03. Therefore, the polysaccharide has a disaccharide repeating unit containing one residue each of D-GlcNAc, D-GlcNAc3*R*lac, and L-Ala.

The ^{1}H and ^{13}C NMR spectra of the polysaccharide were assigned using a set of two-dimensional $^{1}H,^{1}H$ COSY, TOCSY, ROESY, and $^{1}H,^{13}C$ HSQC experiments (table). As the H-1 signals of GlcNAc (G) and GlcNAc3*R*lac (M) were overlapped and poorly resolved, their β-configuration was inferred by the chemical shifts δ_{H-1} 4.50 and 4.52, δ_{C-5} 75.9 and 76.2, respectively (compare published data δ_{C-5} 72.8 and 77.2 for α- and β-Glc*p*NAc, respectively [13]). The configuration of the glycosidic linkages was confirmed by intra-residue H-1/H-3 and H-1/H-5 correlations in the ROESY spectrum, which showed the 1,3-diaxial orientation of these proton pairs.

The position of *R*lac at O-3 of unit M was determined by *R*lac H-2/M H-2 and *R*lac H-2/M H-3 correlations at δ 4.39/3.79 and 4.39/3.63, respectively, in the ROESY experiment, and was confirmed by a relatively low-field position of the signal for M C-3 at δ 80.2 (compare δ 73.3 for G C-3). A low-field position of the signals for C-4 of units G and M at δ 80.8 and 76.2, respectively, as compared with their position in non-substituted β-Glc*p*NAc at δ 71.2 [13], demonstrated the mode of glycosylation of the monosaccharides, which is in agreement with the methylation analysis data (see above). In GlcNAc3*R*lac M, a positive α-effect of glycosylation on C-4 is reduced by a negative β-effect of alkylation by *R*lac at the neighboring O-3. The sequence of units M and G

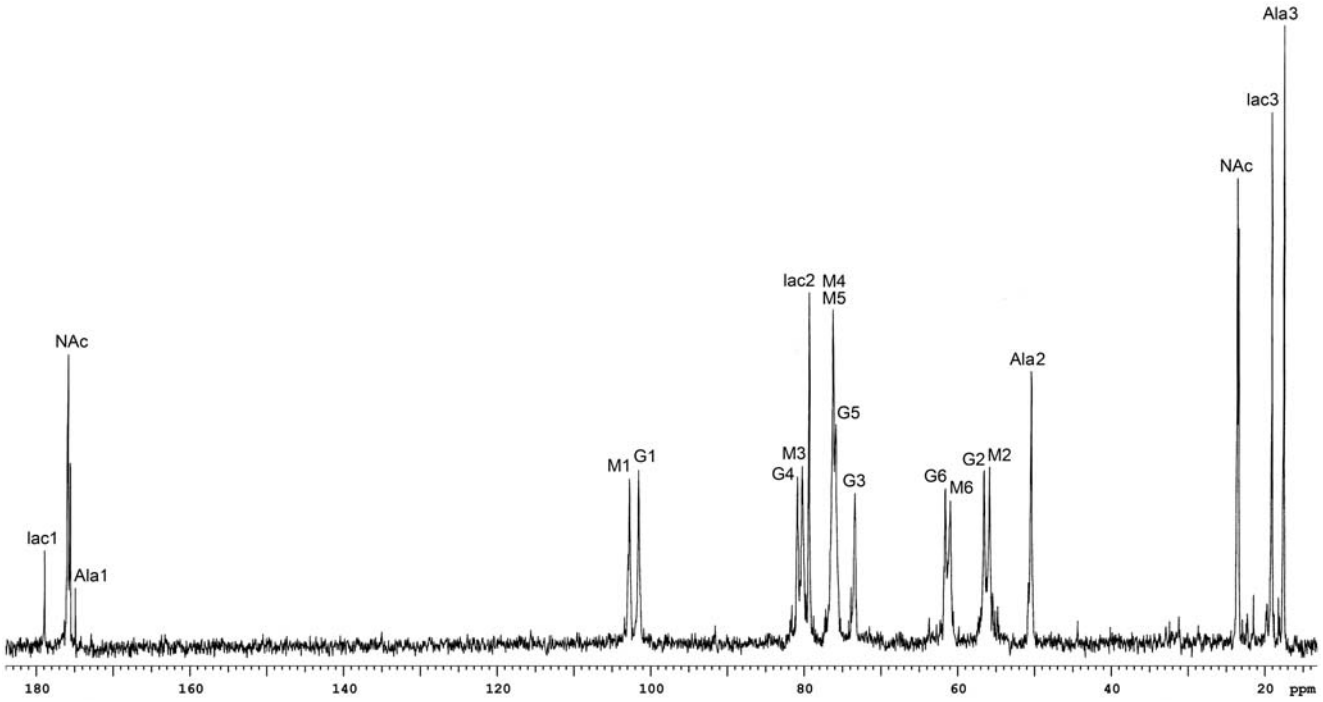

Fig. 1. ^{13}C NMR spectrum of the polysaccharide from *P. alcalifaciens* O45. Arabic numerals refer to carbons in sugar and acid residues denoted by letters as shown in table.

^{1}H and ^{13}C NMR data of the polysaccharide from *P. alcalifaciens* O45 (δ, ppm)

Residue	C-1 *H-1*	C-2 *H-2*	C-3 *H-3*	C-4 *H-4*	C-5 *H-5*	C-6 *H-6a,6b*	*NH-2**
→4)-β-D-Glc*p*NAc-(1→ **G**	101.5 *4.50*	56.5 *3.75*	73.3 *3.68* (−1.8) DD −1.3 ± 0.4 DL +0.3 ± 0.6	80.8 *3.53* (+9.6) DD +9.8 ± 1.4 DL +7.9 ± 1.2	75.9 *3.46*	61.5 *3.68, 3.84*	*8.38*
→3,4)-β-D-Glc*p*NAc-(1→ **M**	102.6 *4.52* (+6.4) DD +6.8 ± 0.7 DL +7.5 ± 0.5	55.8 *3.79*	80.2 *3.63*	76.2 *4.85*	76.2 *3.50*	60.9 *3.69, 3.87*	*7.94*
*R*lac	178.8	79.3 *4.39*	18.9 *1.37*				
L-Ala	174.7	50.4 *4.30*	17.4 *1.43*				*8.32*

Note: Chemical shifts for the *N*-acetyl groups are δ_H 2.03, 1.98; δ_C 23.2, 23.4 (both CH_3), 175.7, and 175.4 (both CO). The experimental glycosylation effects taken as a difference between the chemical shifts of β-Glc*p*NAc in the polysaccharide and free β-Glc*p*NAc [13] are given in parentheses. DD and DL indicate the glycosylation effects in the β-Glc*p*NAc-(1→4)-Glc*p*NAc disaccharide composed of the monosaccharide residues with the same and different absolute configurations, respectively [11].

* Measured in a 9 : 1 H_2O/D_2O mixture.

in the polysaccharide was confirmed by interresidue M H-1/G H-4 and G H-1/M H-4 cross-peaks at δ 4.52/3.53 and 4.50/4.85, respectively, in the ROESY spectrum.

The ^{1}H NMR spectrum of the polysaccharide measured in a 9 : 1 H_2O/D_2O mixture showed signals for three NH protons. Using two-dimensional COSY and one-dimensional TOCSY experiments, two from them were assigned to NH-2 of units M and G, respectively. In the two-dimensional ROESY spectrum (Fig. 2), they gave correlations with the *N*-acetyl groups at δ 7.94/1.98 and 8.38/2.03, respectively, thus indicating *N*-acetylation of both sugar residues at position 2. The remaining NH signal at δ 8.32 showed correlations with Ala H-2 and H-3 and was assigned to NH-2 of alanine. A strong correlation of *R*lac H-2 with Ala NH-2 at δ 4.39/8.32 suggested that the lactic acid residue is amidated with alanine.

The α-glycosylation effects on C-1 of GlcNAc3-*R*lac M and C-4 of D-GlcNAc G and the β-glycosylation effect on C-3 of D-GlcNAc G (+6.4, +9.6 and −1.8 ppm, respectively) indicated the same absolute configuration of the linked monosaccharide residues, i.e. the D-configuration of GlcNAc3*R*lac (compare published data [11] for the same and different absolute configurations (table)).

These data together showed that the polysaccharide from *P. alcalifaciens* O45 is a linear β-(1→4)-linked polymer of GlcNAc and an amide of *N*-acetylmuramic acid with L-alanine (Fig. 3). Earlier, *N*-acetylmuramic acid and its *S* isomer, *N*-acetylisomuramic acid, have been found in a number of bacterial polysaccharides, including the O-polysaccharides of *P. rustigianii* O16 [14] and *P. alcalifaciens* O32 [15]. Remarkably, the polysaccharide studied is structurally related to the bacterial cell wall peptidoglycan [16]; indeed, it has the identical glycan chain, and L-alanine linked to *N*-acetylmuramic acid corresponds to the first amino acid of the stem peptides in peptidoglycan.

Oligosaccharide fractions A and B were studied by negative ion high-resolution ESI MS. The mass spectrum of fraction B showed a molecular peak for a major $Hex_2HexA_1Hep_3Kdo_1Ara4N_1P_1PEtN_3$ oligosaccharide (where Ara4N indicates 4-amino-4-deoxyarabinose, Hep – heptose, Hex – hexose, HexA – hexuronic acid, Kdo – 2-keto-3-deoxyoctonic acid, PEtN – phosphoethanolamine) (experimental and calculated molecular masses 1876.47 and 1876.44 Da, respectively). There were also peaks for two minor oligosaccharides having one less or one more PEtN group. A core oligosaccharide isolated from the LPS of *P. alcalifaciens* O36 has the same composition [17]. Therefore, fraction B represents a core oligosaccharide derived from the R-form LPS devoid of any O-antigen.

The ESI mass spectrum of fraction A demonstrated two major and one minor molecular peaks for the cyclic

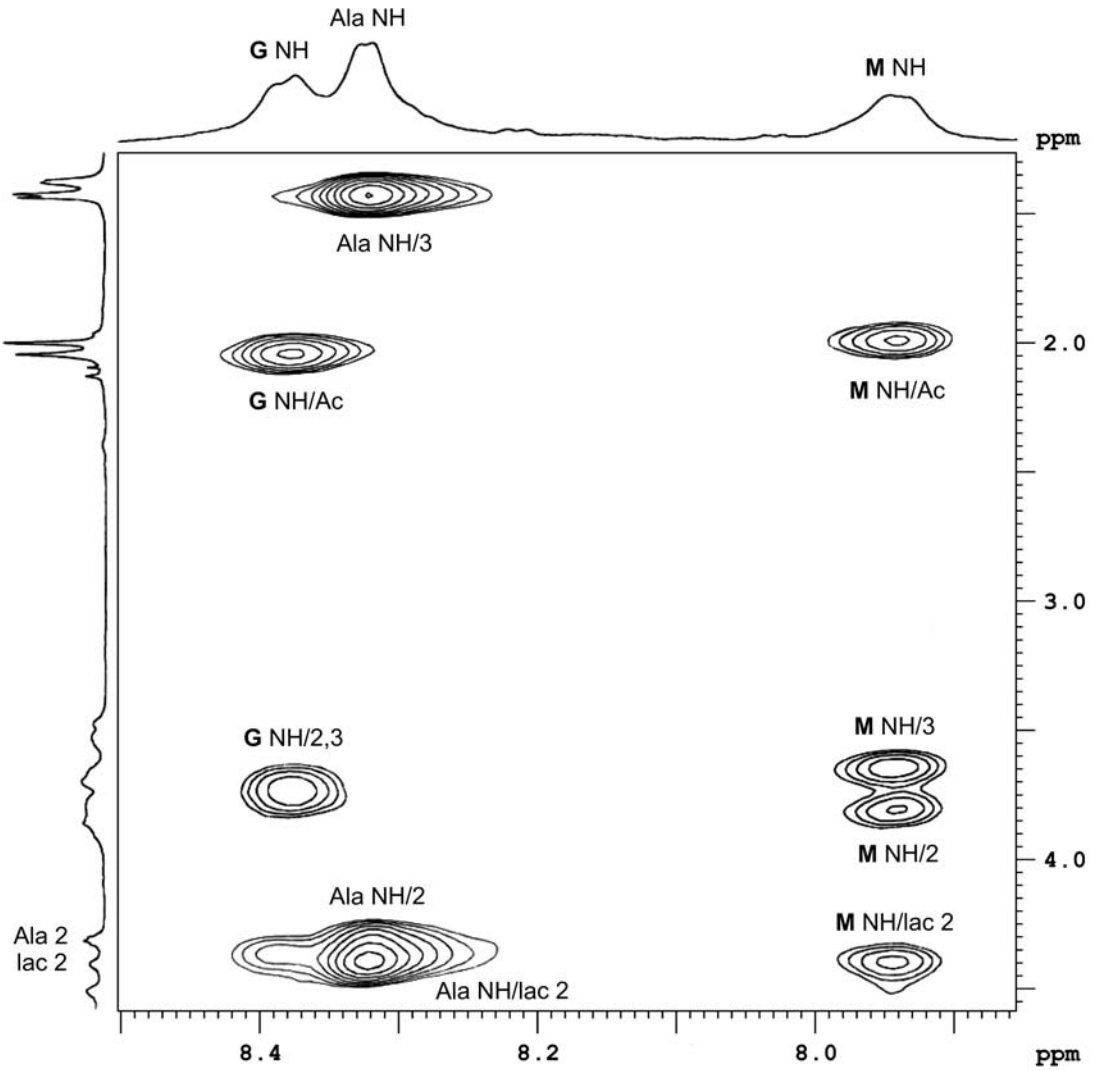

Fig. 2. Part of a ROESY spectrum of the polysaccharide from *P. alcalifaciens* O45 measured in a 9 : 1 H_2O/D_2O mixture. The corresponding parts of the ^1H NMR spectrum are shown along the axes. Arabic numerals refer to protons in sugar and acid residues denoted by letters as shown in table.

dodecasaccharide enterobacterial common antigen bearing from 2 to 4 acetyl groups (2344.91, 2386.92, and 2428.93 Da, respectively) [18]. No oligosaccharide was observed that would correspond to the core bearing one O-unit derived from the SR-type LPS, which usually accompanies the R- and S-type LPS in *Providencia* strains [19]. Instead, a set of minor molecular peaks for compounds of 921.39, 1470.62, 2019.85, and 2569.07 Da were present in the mass spectra of fractions A and B. The mass difference of 549.23 Da between each pair of the peaks corresponds to the $GlcNAc_2$*R*lac_1Ala_1 disaccharide repeat of the *P. alcalifaciens* O45 polysaccharide (the calculated molecular mass 549.22 Da). Hence, the polysaccharide is not linked to the core-lipid A moiety of the LPS but could well be a CPS linked to another kind of lipid.

GLC-MS analysis of the esters derived after methanolysis of the polysaccharide followed by acetylation revealed the major C16:0 fatty acid and traces of C14:0, 3OH-C14:0, and C18:0 fatty acids. The C16:0 fatty acid is a component of *Providencia* lipid A but its content is less than the content of C14:0 and 3OH-C14:0 [20, 21]. The presence of C16:0 as the only dominant fatty acid suggested its role in anchoring the putative CPS into the outer membrane; however, the full structure of the lipid moiety remains to be determined.

NMR spectroscopy screening showed that the studied polysaccharide is not unique for *P. alcalifaciens* O45 but shared by *P. alcalifaciens* O3, O24, and O38. In serogroups O24 and O38, it was the only isolated glycopolymer, whereas in serogroup O3, a long-chain LPS with a neutral O-polysaccharide is produced too (its

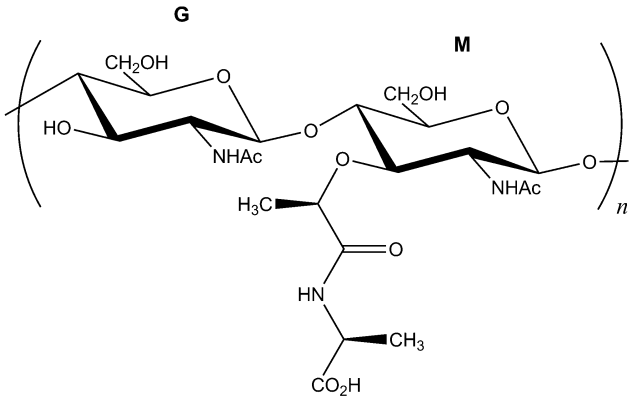

Fig. 3. Structure of the peptidoglycan-related polysaccharide from *P. alcalifaciens* O45.

structure will be reported elsewhere). Recently, one more polysaccharide not associated with the LPS and suggested to be a CPS too has been isolated from *P. rustigianii* O11 [20]. It contains derivatives of 2-amino-2-deoxygalacturonic acid and 2,4-diamino-2,4,6-trideoxyglucose (bacillosamine), which have not been found in *Providencia* O-antigens. In both *P. alcalifaciens* O45 and *P. rustigianii* O11, the putative CPSs are the only serotype-specific surface polysaccharides.

Molecular analyses of gene clusters responsible for the O-antigen biosynthesis in a number of *Providencia* strains revealed the presence of conserved *wza*, *wzb*, and *wzc* genes characteristic for CPS gene clusters [5] and thus demonstrated an association between the LPS and CPS [22]. In *Escherichia coli*, groups 1 and 4 CPS can be expressed on the cell surface in two forms anchoring into the membrane by means of either glycerol phospholipid or the core-lipid A moiety, the latter giving rise to the so-called K_{LPS} [5]. Some *E. coli* strains coexpress K_{LPS} and a "normal" LPS, whereas others produce K_{LPS} as the only serotype-specific polysaccharide. A similar situation seems to occur with the studied *Providencia* strains.

Sequencing and analysis of putative O-antigen gene clusters between the *cpxA* and *yibK* genes in *P. alcalifaciens* O45 and O38 did reveal a set of polysaccharide biosynthesis genes but they did not conform to the structure established. Particularly, putative genes for the synthesis of GDP-L-fucose and UDP-D-glucuronic acid were found in the O45 gene cluster and homologs of the *neuABCD* genes for the synthesis of CMP-*N*-acetylneuraminic acid in the O38 gene cluster, while the corresponding monosaccharides are not components of the polysaccharide produced by these strains. Therefore, we suggest that in *P. alcalifaciens* O45 and O38, the O-antigen gene clusters between *cpxA* and *yibK* are inactivated and instead of the O-antigens, the peptidoglycan-related polysaccharide is produced, whose expression on the cell surface is sufficient for survival of these clones. This resembles the case of *Pseudomonas aeruginosa* O14 and O15 strains, which possess cryptic O-antigen gene clusters or a remnant thereof at the same location on the chromosome as the functional gene clusters in the other *P. aeruginosa* O-serogroups [23, 24]. The serospecificity of O14 and O15 strains is defined by different polysaccharides with disaccharide repeating units, the genes responsible for their synthesis residing evidently elsewhere in the genome [25]. It looks likely that in the *P. alcalifaciens* strains studied, the surface peptidoglycan-like polysaccharide and peptidoglycan itself are synthesized by a combined pathway, which is diverged after adding alanine to *N*-acetylmuramic acid.

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