ISSN 0006-2979, Biochemistry (Moscow), 2011, Vol. 76, No. 6, pp. 707-712. © Pleiades Publishing, Ltd., 2011.
Published in Russian in Biokhimiya, 2011, Vol. 76, No. 6, pp. 864-871.

Structure of the O-Polysaccharide of *Providencia alcalifaciens* O25 Containing an Amide of D-Galacturonic Acid with N^{ε}-[(*R*)-1-carboxyethyl]-L-lysine

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Received January 18, 2011
Revision received February 3, 2011

Abstract—An acidic O-polysaccharide was isolated by mild acid degradation of the lipopolysaccharide of *Providencia alcalifaciens* O25 followed by gel-permeation and anion-exchange chromatography. The O-polysaccharide was studied by sugar and methylation analyses along with ^{1}H and ^{13}C NMR spectroscopy, including two-dimensional correlation ^{1}H,^{13}C HMBC, and ^{1}H,^{1}H ROESY experiments both in D_2O and, to detect correlations for NH protons, in a 9 : 1 H_2O/D_2O mixture. An amino acid was isolated from the polysaccharide by acid hydrolysis and identified as N^{ε}-[(*R*)-1-carboxyethyl]-L-lysine ("alaninolysine", *2S*,*8R*-alaLys) by determination of the specific optical rotation and ^{13}C NMR spectroscopy, using the authentic synthetic diastereomers *2S*,*8R*-alaLys and *2S*,*8S*-alaLys for comparison. The structure of the branched tetrasaccharide repeating unit of the O-polysaccharide was established.

DOI: 10.1134/S0006297911060125

Key words: *Providencia alcalifaciens*, O-antigen, lipopolysaccharide, bacterial polysaccharide structure, opine, N^{ε}-[(*R*)-1-carboxyethyl]-L-lysine

Providencia is a genus of Gram-negative bacteria within the Enterobacteriaceae family. It consists of eight known species [1, 2], among which *P. stuartii*, *P. alcalifaciens*, *P. rustigianii*, and *P. rettgeri* are the most common *Providencia* species that cause human infections. *Providencia* is an opportunistic pathogen associated with traveler's diarrhea [3], foodborne gastroenteritis [4], and urinary tract infections [5], particularly in patients with long-term indwelling urinary catheters or extensive severe burns. The combined serological scheme of *P. stuartii*, *P. alcalifaciens*, and *P. rustigianii* used in epidemiology is based on O-antigens and flagella H-antigens and includes 63 O-serogroups and 30 H-serogroups [1]. The O-antigen (the O-polysaccharide) is a part of the lipopolysaccharide presented on the outer membrane of the cell wall and considered as a virulence factor of Gram-negative bacteria, including *Providencia*. The O-polysaccharide largely contributes to the antigenic variations of the bacterial cell surface, serves as a receptor for bacteriophages, and plays an important role in the adaptive immune response of the host.

The structures of more than 30 *Providencia* O-polysaccharides have been established with the aim of elucidation of the molecular basis of the serological classification of *Providencia* strains (see Bacterial Carbohydrate Structure Database at http://www.glyco.ac.ru/bcsdb3). Most O-polysaccharides are acidic heteropolymers, and many of them contain various unusual monosaccharides and non-sugar components including amino acids, such as L-alanine [6], L-serine [7, 8], D- and L-aspartic acid [9, 10], as well as amino acid derivatives of the opine

Abbreviations: COSY, correlation spectroscopy; GalA, galacturonic acid; GalNAc, N-acetylgalactosamine; GlcA, glucuronic acid; GlcNAc, N-acetylglucosamine; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single-quantum coherence; ROESY, rotating-frame nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy.

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group: N^{ε}-[(*R*)- and (*S*)-1-carboxyethyl]-L-lysine ("alaninolysine", *2S*,*8R*- and *2S*,*8S*-alaLys) [11-13], and *N*-(1-carboxyethyl)alanine (alanopine) [14]. Now we report on a new structure of the O-polysaccharide of *P. alcalifaciens* O25 containing an amide of D-galacturonic acid with *2S*,*8R*-alaLys.

MATERIALS AND METHODS

Bacterial strain, cultivation, and isolation of the lipopolysaccharide. *Providencia alcalifaciens* O25:K2:H4, strain 5350/50 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.

The lipopolysaccharide was isolated in a yield of 9.6% of dry bacterial weight by phenol–water extraction [15] followed by dialysis of the extract without layer separation and freed from insoluble contaminations by centrifugation. The resultant solution was treated with cold (4°C) aqueous 50% CCl_3CO_2H; after centrifugation the supernatant was dialyzed against distilled water and lyophilized.

Isolation of the O-polysaccharide. A portion of the lipopolysaccharide (200 mg) was heated with 2% AcOH for 3 h at 100°C, a lipid precipitate was removed by centrifugation, and the carbohydrate-containing supernatant was fractionated on a column (60 × 2.5 cm) of Sephadex G-50 Superfine in 0.05 M pyridine acetate buffer, pH 4.5, to give a crude polysaccharide in a yield 14% of the lipopolysaccharide mass. The crude polysaccharide was further purified on a DEAE-Trisacryl M column (20 × 1.3 cm) equilibrated with 0.005 M sodium phosphate buffer, pH 6.3, and eluted stepwise with 0.1, 0.25, and 0.5 M sodium phosphate buffers, pH 6.3. The major fraction eluted with 0.1 M sodium phosphate buffer was desalted on a column (80 × 1.6 cm) of TSK HW-40 in water and used for structural studies.

Sugar analysis and isolation and identification of N^{ε}-[(*R*)-1-carboxyethyl]-L-lysine. A polysaccharide sample was hydrolyzed with 2 M CF_3CO_2H (120°C, 2 h) and the solution was evaporated in vacuum. Uronic acids were analyzed using a Biotronik LC2000 sugar analyzer (Germany) equipped with a column (10 × 0.4 cm) of a Durrum DA×8 anion-exchange resin in 0.3 M sodium borate buffer, pH 7.7. Amino components were analyzed by descending paper chromatography on Filtrak FN-11 paper (Germany) using a solvent system (A) of ethyl acetate–pyridine–acetic acid–water (5 : 5 : 1 : 3 v/v, respectively) and detection with ninhydrin. N^{ε}-(1-Carboxyethyl)-L-lysine was isolated by preparative paper chromatography on Filtrak FN-18 paper in the same solvent system in a yield 15% of the polysaccharide weight. Specific optical rotation of the amino acid was measured on a Jasco DIP-360 polarimeter (Japan) in water at 20°C. For NMR spectroscopy, the amino acid was converted into the NH_4-salt by absorption on an Amberlite IR-120 (H^+-form) resin followed by elution with aqueous 5% ammonia.

For determination of the absolute configuration of the monosaccharides [16], a polysaccharide sample was hydrolyzed with 2 M CF_3CO_2H as above and treated with aqueous 12% ammonia (60°C, 1 h) to cleave glucuronolactone. The products were N-acetylated (60 μl Ac_2O in 400 μl aqueous saturated $NaHCO_3$, 0°C, 1 h), subjected to 2-octanolysis (100 μl (*S*)-2-octanol, 15 μl CF_3CO_2H, 120°C, 16 h), acetylated with a 1 : 1 Ac_2O–pyridine mixture (100°C, 1 h), and analyzed using an Agilent Technologies 7820A GC system equipped with a HP-5ms column (Agilent) using a temperature gradient of 160°C (2 min) to 290°C at 7°C/min.

Methylation analysis. The polysaccharide was methylated by the Hakomori procedure [17] and the products were recovered using a Sep-Pak cartridge and divided into two parts, one of which was reduced with $LiBH_4$ in aqueous 70% 2-propanol (20°C, 2 h). Partially methylated monosaccharides were derived by hydrolysis with 2 M CF_3CO_2H, converted into the alditol acetates, and analyzed by GLC-MS on a Kristall 5000M instrument (Khromatek, Russia) equipped with a VF-5MS column (Varian) using the same conditions as in sugar analysis.

NMR spectroscopy. A polysaccharide sample was lyophilized twice from 99.9% D_2O solution and dissolved in 99.95% D_2O. ^1H and ^{13}C NMR spectra were recorded at 30°C on a Bruker AV600 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 for calibration. Two-dimensional NMR spectra were obtained using standard Bruker software, and the Bruker TopSpin 2.1 program was used to acquire and process the NMR data. A mixing time of 200 and 150 msec was used in ROESY and TOCSY experiments, respectively.

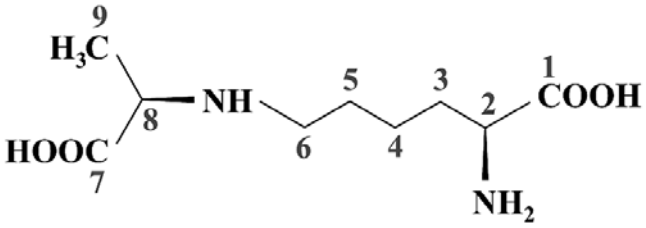
RESULTS AND DISCUSSION

The lipopolysaccharide was isolated from dry bacterial cells by the phenol–water procedure and degraded under mild acidic conditions. The subsequent fractionation of the carbohydrate portion by gel-permeation chromatography on Sephadex G-50 resulted in a crude polysaccharide, which was further purified by anion-exchange chromatography on DEAE-Trisacryl M. Composition analyses of the polysaccharide after full acid hydrolysis revealed two uronic acids (GlcA and GalA), which were detected using a sugar analyzer. Paper chromatography in system A showed the presence of two amino sugars (GlcN, GalN) and an additional amino component hav-

ing the same R_f value as N^{ε}-(1-carboxyethyl)lysine (R_f 0.31 relative to GlcN). Determination of the absolute configuration of the monosaccharides by GLC of the acetylated (+)-2-octyl glycosides showed that all of the monosaccharides have the D-configuration.

The unusual amino component was isolated from the polysaccharide hydrolysate by descending paper chromatography and identified as N^{ε}-[(*R*)-1-carboxyethyl)-L-lysine (*2S*,*8R*-alaLys; Scheme 1). The optical rotation value $[\alpha]_D$ +9.4° (*c* 0.36, water) showed that the lysine moiety in the amino acid has the L-configuration (compare published data $[\alpha]_D$ +9.7° and +11.6° for N^{ε}-[(*R*)-1-carboxyethyl)-L-lysine and N^{ε}-[(*S*)-1-carboxyethyl)-L-lysine, respectively [18]). The structure of *2S*,*8R*-alaLys was confirmed, and the (*R*)-configuration of the 1-carboxyethyl group (the alanine moiety) was established by ^{13}C NMR spectroscopy as described [12, 19] using authentic samples of *2S*,*8R*-alaLys and *2S*,*8S*-alaLys for comparison.

Linkage analysis by GLC–mass spectrometry of the partially methylated alditol acetates derived from the methylated polysaccharide revealed 3-substituted and 4,6-disubstituted hexosamines. In addition to these monosaccharides, a similar analysis after carboxyl-reduction of the methylated polysaccharide resulted in identification of a 4,6-disubstituted hexose, which was derived from a 4-substituted uronic acid. Although the polysaccharide is branched, no terminal monosaccharide derivative was detected. Therefore, it was suggested that the terminal position of the side chain is occupied by a uronic acid, which was not carboxyl-reduced due to its amidation by *2S*,*8R*-alaLys (see below).



Structure and atom numeration of N^{ε}-[(*R*)-1-carboxyethyl]-L-lysine

Scheme 1

The ^{13}C NMR spectrum of the polysaccharide demonstrated a tetrasaccharide repeating unit. It contained signals for four anomeric carbons at δ 101.0-104.3, two nitrogen-bearing carbons at δ 55.8 and 53.3 (C2 of GlcN and GalN), two HO*C*H_2–C groups (C6 of GlcN and GalN), from which one was O-substituted (δ 66.3) and the other non-substituted (δ 62.0), one free carboxyl group at δ 174.6 (C6 of GlcA), one carboxamide group at δ 171.9 (C6 of GalA) and two *N*-acetyl groups at δ 23.8, 23.9 (both CH_3), 175.7, and 176.3 (both CO). There were no signals in the region δ 82-88 characteristic for C4 of furanosides [20] (that at δ 83.6 was assigned later to C3 of GlcN); hence, all sugar residues are pyranosidic. The ^1H NMR spectrum of the polysaccharide (Fig. 1) showed

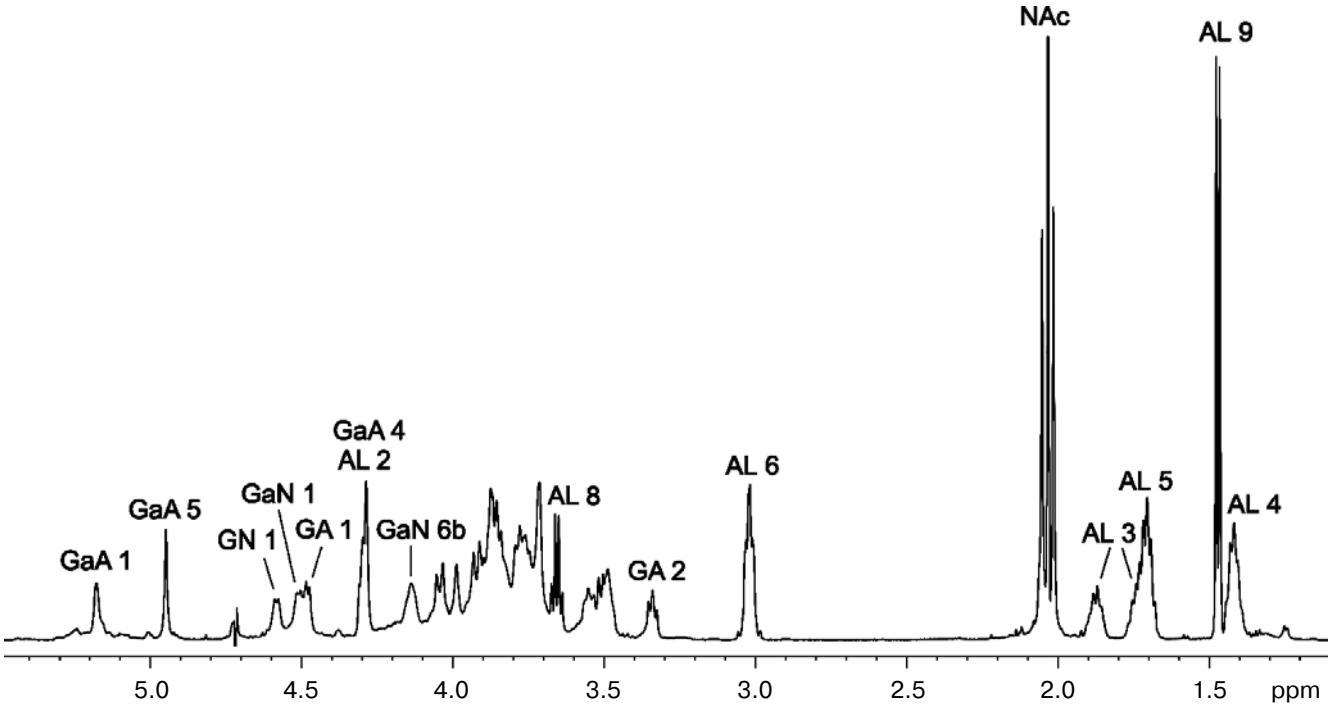


Fig. 1. ^1H NMR spectrum of the O-polysaccharide from *P. alcalifaciens* O25. Arabic numerals refer to protons in N^{ε}-(1-carboxyethyl)lysine and sugar residues denoted as follows: AL, N^{ε}-(1-carboxyethyl)lysine; GN, GlcN; GaN, GalN; GA, GlcA; GaA, GalA.

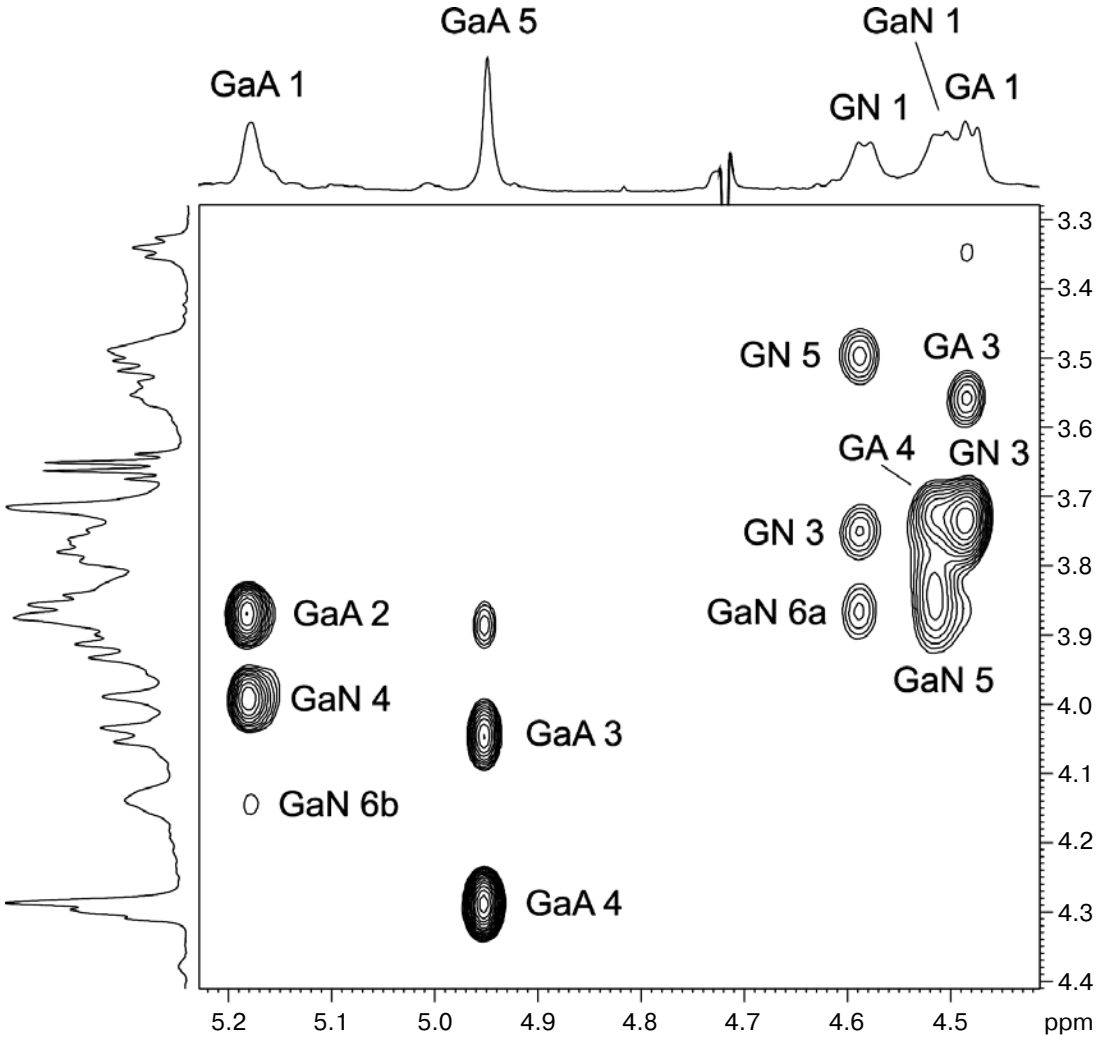


Fig. 2. Part of a two-dimensional ROESY spectrum of the O-polysaccharide from *P. alcalifaciens* O25 measured in D_2O. The corresponding parts of the ^1H NMR spectrum are shown along the axes. Arabic numerals refer to protons in *N*$^{\varepsilon}$-(1-carboxyethyl)lysine and sugar residues denoted as indicated in the legend to Fig. 1.

signals for four anomeric protons at δ 4.48-5.17, two *N*-acetyl groups at δ 2.01 and 2.05, and other protons in the region δ 3.35-4.95. The NMR spectra also contained signals having a non-sugar origin at δ_H 1.42, 1.48, 1.71, 1.74, 1.88, 3.02, 3.66, 4.30 and δ_C 16.3, 23.4, 26.7, 32.6, 47.1, 55.4, 58.9, 176.0, 179.0, whose chemical shifts were essentially the same as those of *N*$^{\varepsilon}$-(1-carboxyethyl)lysine in the O-polysaccharides of *P. rustigianii* O14 [13] and *P. alcalifaciens* O23 [11].

The ^1H and ^{13}C NMR spectra of the O-polysaccharide were assigned using two-dimensional homonuclear $^1H,^1H$ COSY, TOCSY, ROESY, and heteronuclear H-detected $^1H,^{13}C$ HSQC and HMBC experiments (table). The COSY and TOCSY spectra revealed spin systems for two sugar residues having the *gluco* configuration (GlcN and GlcA) and two residues with the *galacto* configuration (GalN and GalA). As judged by $J_{1,2}$ coupling constants of ~7 Hz, GlcN, GlcA, and GalN are β-linked, whereas GalA is α-linked ($J_{1,2}$ < 3 Hz).

The signals for GlcN C3, GlcA C4, and GalN C4 and C6 were shifted downfield to δ 83.6, 81.2, 76.1, and 66.3, as compared with their positions in the corresponding non-substituted monosaccharides at δ 74.81, 72.69, 68.85, and 61.89, respectively [21]. The ^{13}C NMR chemical shifts for C2-C5 of GalA were close to those of the non-substituted monosaccharide [21]. These findings confirmed the methylation analysis data and showed that the polysaccharide is branched with a lateral GalA residue and a 4,6-disubstituted GalN residue at the branching point. Sequence analysis of the polysaccharide was performed using a ROESY experiment in D_2O (Fig. 2), which showed interresidue cross-peaks between the following anomeric protons and protons at the linkage carbons: GalA H1/GalN H4 at δ 5.17/4.00; GalN

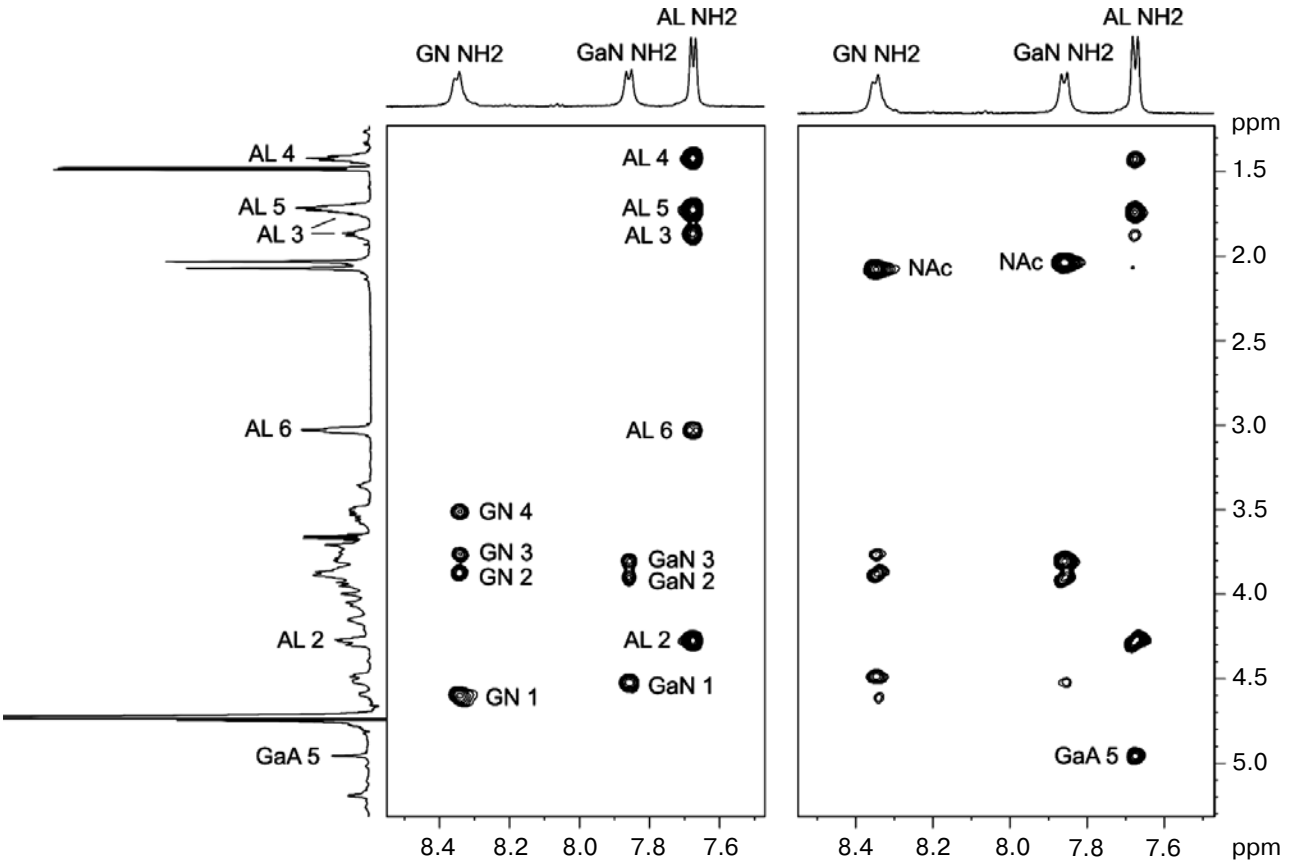
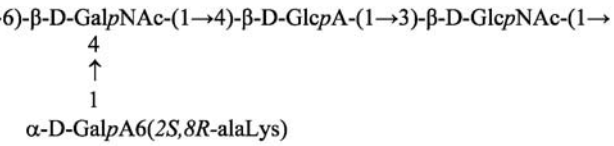


Fig. 3. Parts of two-dimensional TOCSY (left) and ROESY (right) spectra of the O-polysaccharide from *P. alcalifaciens* O25 measured in a 9 : 1 H_2O/D_2O mixture. The corresponding parts of the ^1H NMR spectrum are shown along the axes. Arabic numerals refer to protons in N^{ε}-(1-carboxyethyl)lysine and sugar residues denoted as indicated in the legend to Fig. 1.

^1H and ^{13}C NMR chemical shifts of the O-polysaccharide of *P. alcalifaciens* O25 (δ, ppm). Chemical shifts for the *N*-acetyl groups are δ_H 2.01 and 2.05, δ_C 23.8, 23.9 (both CH_3), 175.7 and 176.3 (both CO)

Residue	C1 *H1*	C2 *H2*	C3 *H3 (3a, 3b)*	C4 *H4*	C5 *H5*	C6 *H6 (6a, 6b)*	C7 *H7*	C8 *H8*	C9 *H9*	*NH2*[a]
→3)-β-D-Glc*p*NAc-(1→	101.8 *4.58*	55.8 *3.86*	83.6 *3.75*	69.8 *3.52*	76.6 *3.50*	62.0 *3.78, 3.93*				*8.35*
→4)-β-D-Glc*p*A-(1→	104.3 *4.48*	73.8 *3.35*	74.9 *3.56*	81.2 *3.72*	77.5 *3.72*	174.6				
→4,6)-β-D-Gal*p*NAc-(1→	102.7 4.52	53.3 3.89	71.3 3.79	76.1 4.00	73.2 3.83	66.3 3.89, 4.14				*7.86*
α-D-Gal*p*A-(1→	101.0 *5.17*	69.6 *3.86*	70.3 *4.05*	71.0 *4.29*	72.6 *4.95*	171.9				
2S,8R-alaLys	179.0	55.4 *4.30*	32.6 *1.74, 1.88*	23.4 *1.42*	26.7 *1.71*	47.1 *3.02*	176.0	58.9 *3.66*	16.3 *1.48*	*7.68*

[a] Measured in a 9 : 1 H_2O/D_2O mixture.



Structure of the repeating unit of O-polysaccharide from *P. alcalifaciens* O25. GalA6(*2S*,*8R*-alaLys) stands for N^{α}-(D-galacturonoyl)-N^{ε}-[(*R*)-1-carboxyethyl]-L-lysine

Scheme 2

H1/GlcA H4 at δ 4.52/3.72; GlcA H1/GlcN H3 at δ 4.48/3.75; GlcN H1/GalN H6a at δ 4.58/3.89.

The structure of *2S*,*8R*-alaLys was confirmed by the $^{1}H,^{13}C$ HMBC spectrum, which showed correlations between the lysine and the alanine moieties: H6/C8 at δ 3.02/58.9 and C6/H8 at δ 47.1/3.66. Acylation of *2S*,*8R*-alaLys at N2 by the carboxyl group of GalA was inferred from: i) a correlation between *2S*,*8R*-alaLys NH2 and GalA H5 at δ 7.68/4.95 in the ROESY spectrum measured in a 9 : 1 H_2O/D_2O mixture (Fig. 3); ii) the lack of pD dependence of the chemicals shifts of H5 and C5 of GalA, whereas those of GlcA shifted from δ_H 3.72 and δ_C 77.5 to δ_H 3.90 and δ_C 75.8, respectively, upon a pD change from 3 to 1, and iii) an upfield position of the C6 signal of GalA at δ 171.9, which is characteristic for hexuronamides (compare published value δ 176.43 for C6 of Na-salt of α-GalA [21]).

Therefore, the O-polysaccharide of *P. alcalifaciens* O25 has the structure shown in Scheme 2. A peculiar feature of the studied polysaccharide is the presence of *2S*,*8R*-alaLys, an amino acid derivative of the opine family [22]. Its lysine moiety has the L-configuration as in all stereoisomers of N^{ε}-(1-carboxyethyl)lysine found so far in bacterial polysaccharides, whereas the alanine moiety may be either D or L. Besides *Providencia* [11-13], *2S*,*8R*-alaLys or *2S*,*8S*-alaLys linked to either GalA or GlcA have been identified earlier in the O-polysaccharides of bacteria *Proteus mirabilis* O13 [23] and *Proteus myxofaciens* O60 [24] closely related to *Providencia* as well as in a taxonomically remote marine bacterium *Shewanella fidelis* KMM 3582^{T} [25].

This work was supported by the Russian Foundation for Basic Research (project No. 08-04-91221-NNSF).

We thank N. P. Arbatsky for help with sugar analysis.

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