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Structure of an acidic polysaccharide from the marine bacterium *Pseudoalteromonas aliena* type strain KMM 3562^T containing two residues of L-serine in the repeating unit

Eva-Lisa Nordmark,[a] Andrei V. Perepelov,[a,b] Alexander S. Shashkov,[b] Evgeny L. Nazarenko,[c] Raisa P. Gorshkova,[c] Elena P. Ivanova[c] and Göran Widmalm[a,*]

[a]*Department of Organic Chemistry, Arrhenius Laboratory, Stockholm University, S-106 91 Stockholm, Sweden*
[b]*N.D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow 119991, Russian Federation*
[c]*Pacific Institute of Bioorganic Chemistry, Far East Branch of the Russian Academy of Sciences, Vladivostok 690022, Russian Federation*

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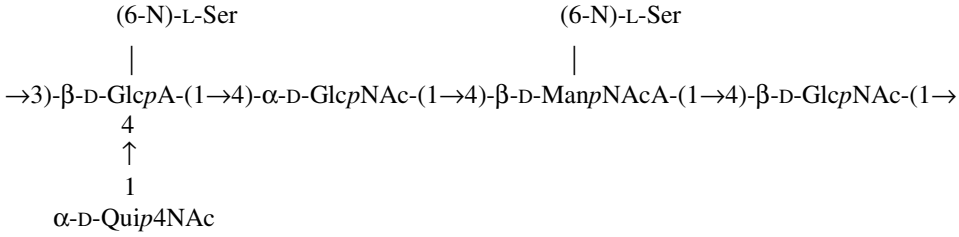
Abstract—The structure of an acidic polysaccharide from *Pseudoalteromonas aliena* type strain KMM 3562^T has been elucidated. The polysaccharide was studied by component analysis, ^{1}H and ^{13}C NMR spectroscopy, including 2D NMR experiments. A ^{1}H, ^{13}C band-selective constant-time heteronuclear multiple-bond connectivity experiment was used to determine amide linkages, between serine and uronic acid (UA) residues, via $^3J_{H,C}$ correlations between Ser–αH and UA–C-6. It was found that the polysaccharide consists of pentasaccharide repeating units with the following structure:



Keywords: *Pseudoalteromonas aliena*; Polysaccharides; Structure; NMR spectroscopy; Marine bacteria; 2-Acetamido-2-deoxy-D-mannuronic acid; 4-Acetamido-4,6-dideoxy-D-glucose; Serine

1. Introduction

Ubiquitous *Alteromonas*-like marine proteobacteria have been intensively studied over the last years.[1,2] These heterotrophic organisms are constantly in the focus of research interests of microbiologists, systematics, biochemists, and biotechnologists due to their taxonomic diversity and metabolic activities such as the production of various biologically active compounds.[3–5] Currently, *Alteromonas*-like bacteria are divided into several families, namely Alteromonadaceae, Pseudoalteromonadaceae, Colwelliaceae, Shewanellaceae, Moritellaceae, Ferrimonadaceae, Idiomarinaceae, and Psychromonadaceae.[6] Amongst these taxa, *Pseudoalteromonas* spp. represent the most numerous cluster.

* Corresponding author. Tel.: +46 8 163 742; fax: +46 8 154 908; e-mail: gw@organ.su.se

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During the course of the taxonomic investigations of marine *Proteobacteria*, a collection of *Alteromonas*-like bacteria from the North-West Pacific was studied. Seven strains out of 200 isolates appeared to be phenotypically similar to that of *Pseudoalteromonas distincta*.[7] The description of this species was based on a single strain, KMM 638^T, originally isolated from a marine sponge collected at a depth of 350 m near Komandorskie Islands, Russia. This bacterium was initially affiliated to the genus *Alteromonas* and described as *Alteromonas distincta*, but later on reclassified as *P. distincta*.[8] Further genetic and phylogenetic investigations of seven strains originated from sea water samples collected from a few locations in the Amursky Bay of the Sea of Japan, near Vladivostok city, revealed that these bacteria belong to a separate species, *Pseudoalteromonas aliena*.[7] The type strain of this species, SW19^T (=KMM 3562^T = LMG 22059^T), has been subjected to structure investigation of its acidic antigenic polysaccharide.

2. Results and discussion

The lipopolysaccharide (LPS) was isolated from *P. aliena* type strain KMM 3562^T by the phenol–water procedure.[9] On degradation with dilute acetic acid, the LPS afforded a high molecular weight polysaccharide (PS) isolated using gel permeation chromatography.

Sugar analysis of the polysaccharide using GLC of derived alditol acetates revealed 2-amino-2-deoxy-glucose (GlcN). Moreover, methanolysis of the polysaccharide followed by GLC analysis of acetylated methyl derivatives showed the presence of glucuronic acid (GlcA), 4-amino-4,6-dideoxy-glucose (Qui4N), and an amino acid, serine (Ser). Subsequent GLC analysis of the components derivatized with (*S*)-2-butanol followed by acetylation showed that GlcN, Qui4N, and GlcA have the D configuration and that Ser has the L configuration. The fifth sugar (see below), 2-amino-2-deoxy-mannuronic acid, ManNA, was identified from a number of one- and two-dimensional NMR experiments.

The ^{13}C NMR spectrum of the PS was typical for the regular polymer and showed five signals from the anomeric carbons at 97.4–103.4 ppm indicating a pentasaccharide repeating unit. The spectrum also contained, inter alia, several signals in the nitrogen bearing carbon region, methyl signals characteristic of *N*-acetyl groups, one signal of a methyl group at δ 18.0, and eight signals in the region for carbonyl groups, δ 170.5–176.8. The ^{1}H, ^{13}C HSQC spectrum (Fig. 1) confirmed the presence of five sugars in the repeating unit. The absence from signals for non-anomeric sugar carbons at a lower field than δ 82 in the ^{13}C NMR spectrum demonstrated the pyranoid form of all sugar residues.[10] The five anomeric signals of the PS found in the low-field region of the ^{1}H NMR spectrum at δ 4.54–5.57 were not well resolved

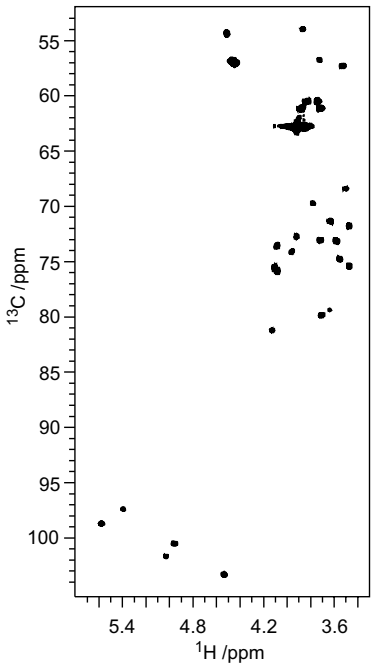


Figure 1. Part of the ^{1}H, ^{13}C HSQC spectrum of the PS from *Pseudoalteromonas aliena* type strain KMM 3562^T.

and the anomeric configuration of the sugar residues was deduced from $^1J_{C,H}$ coupling constants[11] as well as ^{1}H, ^{1}H NOESY cross-peak patterns. The high-field region of the spectrum contained a signal for a methyl group at δ 1.06 of the 6-deoxy-sugar, as well as signals from four *N*-acetyl groups at δ 2.03–2.08. Although the methylation analysis, performed as previously described,[12] only gave 2-deoxy-3,6-di-*O*-methyl-2-*N*-methylacetamido-D-glucose as a component of the polymer, this indicated, together with the above NMR data, the presence of 4-linked D-Glc*p*NAc as a part of the polysaccharide.

The five sugar residues are denoted **A**–**E** in order of the decreasing chemical shift of their anomeric protons (Table 1). For pyranoid sugar residues the difference in $^1J_{C,H}$ coupling constants is ~10 Hz between α- and β-linked sugars showing that residues **A** and **B** are α-linked and that **C**–**E** are β-linked. These findings were further corroborated by ^{1}H, ^{1}H NOESY cross-peaks between H-1 and H-2 in the former and H-1 and H-3/H-5 in the latter. Residue **A** was readily identified, inter alia, from ^{1}H, ^{1}H TOCSY experiments as an α-D-Qui*p*4N residue. From a combination of 2D NMR experiments including ^{1}H, ^{13}C HMBC and ^{1}H, ^{13}C HSQC-TOCSY, residue **B**, with a resonance at δ_C 60.5 from an unsubstituted hydroxymethyl group and a low-field chemical shift due to glycosylation at δ_C 79.5 was identified as a 4-substituted α-D-Glc*p*N residue. However, residue **C**, with $\delta_{C\text{-}6}$ 61.1, had in its spin-system four protons resonating at ~3.72 ppm, making the assignments more problematic. The ^{1}H, ^{13}C HSQC-TOCSY spectrum with

Table 1. ^{1}H and ^{13}C NMR chemical shifts (ppm) of the signals from the polysaccharide of *Pseudoalteromonas aliena* type strain KMM 3562^{T} and inter-residue correlations from anomeric protons in the NOESY spectrum and anomeric atoms in the HMBC spectrum

Sugar residue	1	2	3	4	5	6	Me	CO	α	β	COONa	NOE	HMBC
α-D-Qui*p*4NAc-(1→ **A**	5.57	3.58	3.63	3.52	3.51	1.06	2.07					H-4, **E**	C-4, **E**
	98.8 [177]	73.1	71.3	57.2	68.4	18.0	23.6	176.0					H-4, **E**
→ 4)-α-D-Glc*p*NAc-(1→ **B**	5.40	3.86	3.77	3.63	3.46	3.75, 3.81	2.03					H-4, **D**	(C-4, **D**)[a]
	(0.19)	(−0.02)	(0.02)	(0.14)	(−0.40)								
	97.4 [179]	53.9	69.7	79.5	71.8	60.5	23.3	175.6					H-4, **D**
	(5.6)	(−1.1)	(−2.0)	(8.2)	(−0.7)	(−1.3)							
→ 4)-β-D-Glc*p*NAc-(1→ **C**	5.02	3.72	3.72	3.71	3.47	3.72, 3.86	2.05					H-3, **E**	C-3, **E**
	(0.30)	(0.07)	(0.16)	(0.25)	(0.01)								
	101.8 [167]	56.7	73.0	79.8	75.4	61.1	23.6	175.8					H-3, **E**
	(6.0)	(−1.2)	(−1.8)	(8.7)	(−1.4)	(−0.8)							
→ 4)-β-D-Man*p*NAc*A*-6-*N*-L-Ser-(1→ **D**	4.95	4.51	4.08	3.91	4.09		2.08		4.46	~3.92		H-4, **C**	C-4, **C**
	100.6 [167]	54.3	73.6	72.6	75.8	170.7	23.4	176.8	56.8	62.8	174.2		
→ 3,4)-β-D-Glc*p*A-6-*N*-L-Ser-(1→ **E**	4.54	3.55	4.12	3.96	4.11				4.45	3.86, 3.92		H-4, **B**	C-4, **B**
	(−0.11)	(0.25)	(0.60)	(0.42)	(0.39)								
	103.4 [166]	74.8	81.3	74.2	75.6	170.5			56.9	62.8	175.1		H-4, **B**
	(6.6)	(−0.2)	(4.8)	(1.5)	(−1.3)	(−6.0)							

$J_{H-1,C-1}$ values are given in square brackets. Chemical shift differences as compared to the corresponding monosaccharides are given in parentheses.
[a] Peak overlap with C-5 in **B**.

a short mixing time of 20 ms[13] showed, inter alia, cross-peaks between δ_{H-5} 3.47 and δ_{C-4} 79.8, the latter being the chemical shift of the glycosyloxylated carbon, and between δ_{H-4} 3.71 and δ_{C-5} 75.4, thereby assigning the β-D-Glc*p*N residue as 4-substituted. The two remaining sugar spin-systems from residues **D** and **E** contained resonances for H-1 to H-5 showing the presence of uronic acids. Chemical shifts of, in particular, C-1, C-2, and H-2, together with the additional NOE between H-1 and H-2 and HMBC correlations between H-1 and C-2, H-2 and C-3 as well as C-4, identified residue **D** as β-Man*p*NA. It was evident from a correlation in the HMBC spectrum (see below) that it is 4-substituted. Residue **E** could be assigned to a β-D-Glc*p*A residue. The glycosylation shifts ($\Delta\delta$) of C-3 and C-4 indicate that this is a branch-point sugar residue.[14] The amino groups at C-4 in residue **A** and C-2 in residues **B**–**D** were all shown to be *N*-acetylated as HMBC correlations from carbonyl groups to the corresponding sugar ring protons were observed.

The above described substitution patterns were corroborated and the sequence of sugar residues was elucidated by analysis of ^{1}H, ^{1}H NOESY (Fig. 2) and ^{1}H, ^{13}C HMBC spectra (Table 1). The absolute configuration of ManNAcA was ascertained from examination of the ^{13}C glycosylation effects. The ^{13}C glycosylation shifts ($\Delta\delta$) for C-3, C-4, and C-5 of the 4-substituted β-D-Glc*p*NAc residue were −1.8, +8.7, and −1.4 ppm, respectively. These ^{13}C glycosylation shifts are characteristic of the D absolute configuration of ManNAcA (cf. Table 2 in Ref. 15). The carbohydrate part of the PS consists of pentasaccharide repeating units with the following structure:→3)[α-D-Qui*p*4NAc-(1→4)]-β-D-Glc*p*A-(1→4)-α-D-Glc*p*NAc-(1→4)-β-D-Man*p*NAcA-(1→4)-β-D-Glc*p*NAc-(1→. The chemical shifts of C-6 in the uronic acid residues **D** and **E** indicated that these residues were substituted via an amide linkage,[16] presumably by serine residue detected in the chemical analysis.

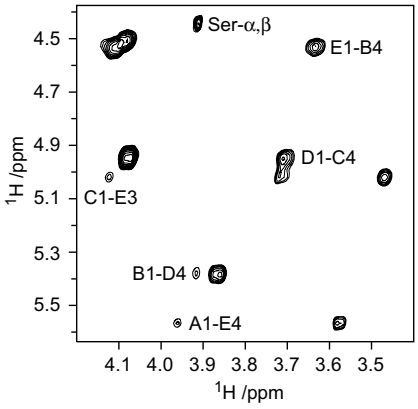
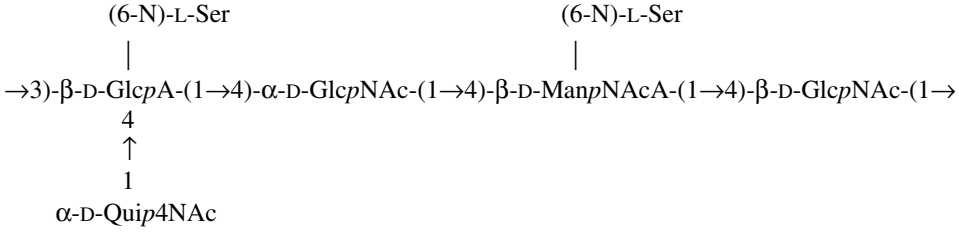


Figure 2. Selected region of the ^{1}H, ^{1}H NOESY spectrum of the PS from *Pseudoalteromonas aliena* type strain KMM 3562^{T}.

The substitution by L-serine residues was indicated by a ^{1}H, ^{1}H NOESY experiment performed in an H_2O/D_2O mixture, which revealed correlations for NH protons. The spectrum showed intense cross-peaks between NH protons of serine residues to H-5 protons of β-D-Man*p*NAcA and β-D-Glc*p*A at δ 8.45/4.09 and 8.30/4.11, respectively. Therefore serine residues should be attached via amide linkages C-6 of residues **D** and **E**, respectively. Moreover, the NOESY spectrum demonstrated correlations between NH protons of residues **A**–**D** with the methyl group of the *N*-acetyl groups at δ_H 7.47/2.06, 7.90/2.02, 7.89/2.04, and 7.96/2.07. To further elucidate the substitution positions of the serine residues analysis of the pD dependence of the ^{1}H NMR chemical shifts in the spectrum was performed. The signals for H-2 of the two Ser residues shifted upfield by ~0.2 ppm, with an increase of pD from 3 to 9; hence, these components had a free carboxyl group.[17] No significant pD dependence was observed for the H-5 signals of ManNAcA and GlcA, thus showing amidation of the carboxyl groups of these residues with the amino groups of the serine residues.

This substitution pattern was confirmed by a ^{1}H, ^{13}C BS-CT-HMBC experiment,[18] with high resolution in the ^{13}C dimension, since the C-6 resonances differ by only 0.2 ppm (Table 1). In the corresponding spectrum $^3J_{H,C}$ correlations are observed between C-6 and Ser–αH as well as H-4, and $^2J_{H,C}$ correlations between C-6 and H-5, in residues **D** and **E** (Fig. 3). Thus, linkages are confirmed and all spin-systems in the PS have been assigned.

These data define the following structure of the polysaccharide from *P. aliena* type strain KMM 3562^T:



To our knowledge, two constituents as amides of uronic acids with L-serine, namely, *N*-(2-acetamido-2-deoxy-D-mannuronoyl)-L-serine and *N*-(Dglucuronoyl)-L-serine have not been found hitherto in nature as part of a polysaccharide. Furthermore, this polymer contains another unusual sugar, 4-acetamido-4,6-dideoxy-D-glucose, and therefore the polysaccharide from *P. aliena* type strain KMM 3562^T is unique among bacterial carbohydrate polymers.

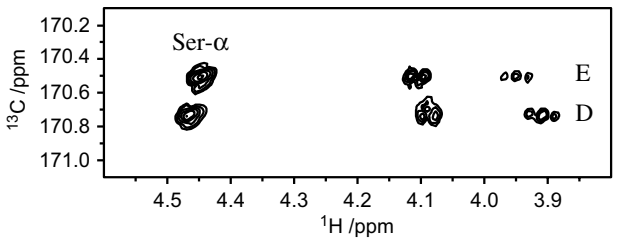


Figure 3. Selected region of the ^{1}H, ^{13}C BS-CT-HMBC spectrum of the PS from *Pseudoalteromonas aliena* type strain KMM 3562^T.

3. Experimental

3.1. Bacterial growth, isolation, and degradation of the lipopolysaccharide

P. aliena type strain KMM 3562^T was grown on the modified Yoshimizu–Kimura medium.[19] Wet bacterial cells were extracted with hot aq 45% phenol, the resulting mixture was centrifuged, the aqueous layer dialyzed, freed from insoluble contaminations by centrifugation, concentrated in vacuum, and freeze-dried to yield 500 mg of the LPS from 20 L of cultural fluid.

Mild acid degradation of the LPS (100 mg) was performed with aq 1% HOAc at 100 °C until precipitation of lipid A occurred (2.5 h). The precipitate was removed by centrifugation (13,000 *g*, 20 min) and the supernatant fractionated by GPC on a column (56 × 2.6 cm) of Sephadex G-50 (S) in 0.05 M pyridinium acetate buffer, pH 4.5, monitored with a Knauer differential refractometer. The yield of the PS was 36% of the LPS weight.

3.2. Chemical analyses

The polysaccharide was hydrolyzed with 2 M CF_3CO_2H (120 °C, 2 h), monosaccharides were reduced with 0.25 M $NaBH_4$ in aq 1 M ammonia (20 °C, 1 h), acetylated with a 1:1 (v/v) mixture of pyridine and acetic anhydride (120 °C, 30 min) and analyzed by GLC. Methanolysis of the polysaccharide (1 mg) was carried out using 1 M HCl–MeOH (85 °C, 16 h), followed by acetylation with Ac_2O in pyridine (120 °C, 30 min), and subsequently analyzed by GLC. The absolute configurations of the monosaccharides were determined by GLC of acetylated (*S*)-(+)-2-butyl glycosides according to published methods.[20] Absolute configuration of serine residues was determined by GLC of acetylated (*S*)-(+)-2-butyl ester using authentic L-serine and D-serine as references. GLC was performed using a Hewlett-Packard 5890 Series II instrument equipped with an

HP fused silica column (0.25 mm × 30 m) using a temperature program of 170–180 °C (1 °C min^{-1}) followed by 180–230 °C (7 °C min^{-1}).

3.3. NMR spectroscopy

Samples were deuterium-exchanged by freeze-drying from D_2O and then examined in solutions of D_2O or an H_2O/D_2O (85:15) mixture, using as internal reference sodium 3-trimethylsilyl-[2,2,3,3^{2}H$_4$]propanoate (δ_H = 0.00) or acetone (δ_H = 2.225) and as external reference dioxane in D_2O (δ_C = 67.4). NMR spectra were recorded for the polysaccharide at 65 °C on Varian Inova 600 and 800 MHz and Bruker 500 MHz spectrometers, unless otherwise stated. The ^{1}H, ^{13}C band-selective constant-time heteronuclear multiple-bond connectivity (BS-CT-HMBC) experiment was performed on a sample containing 18 mg of the PS in 0.6 mL D_2O at 57 °C on a Bruker DRX 500 spectrometer equipped with a Cryoprobe. The $^nJ_{C,H}$ coupling evolution delay (Δ_1) was set to 50 ms and the spectrum was collected with 1024 scans per increment over 7 ppm (^{1}H) × 9 ppm (^{13}C covering the full carbonyl region between 169 and 178 ppm) as 4k × 128 data points, corresponding to a $t_{1(max)}$ of 114 ms. Selection of the carbonyl resonances was achieved with a 0.1 ms ^{13}C 180° Gaussian pulse. Half-sine shaped pulsed field gradient pulses were applied with a 1 ms duration. All other NMR experiments were performed according to standard pulse sequences and data were processed using the software supplied by the manufacturers. The chemical shifts were compared to those of the corresponding monosaccharides.[21]

Acknowledgements

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